CANCER RESEARCH

VOL. 9 JUNE 1949 No. 6

CONTENTS

THE OFFICIAL ORGAN OF THE

AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC.

Published by THE UNIVERSITY OF CHICAGO PRESS

CANCER RESEARCH

This journal is sponsored by The American Association for Cancer Research, Inc.; The Anna Fuller Fund; Cancer Research Division, Donner Foundation, Inc.; The Jane Coffin Childs Memorial Fund for Medical Research; and The Elsa U. Pardee Foundation.

CANCER RESEARCH is published monthly for Cancer Research, Inc., by the University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois. The subscription price is $7.00 per year; the price of single copies is $1.00. Orders for less than a full year will be charged at the single copy rate. Postage is prepaid by the publishers on all orders from the United States and its possessions, Argentina, Bolivia, Brazil, Chile, Colombia, Costa Rica, Cuba, Dominican Republic, Ecuador, Guatemala, Haiti, Republic of Honduras, Mexico, Morocco (Spanish Zone), Nicaragua, Panama, Paraguay, Peru, Rio de Oro, El Salvador, Spain (including Balearic Islands, Canary Islands, and the Spanish Offices in Northern Africa; Andorra), Spanish Guinea, Uruguay, and Venezuela. Postage is charged extra as follows: For Canada and Newfoundland, 48 cents on annual subscriptions (total $7.48), on single copies 4 cents (total $1.04); for all other countries in the Postal Union, $1.20 on annual subscriptions (total $8.20), on single copies 10 cents (total $1.10).

The following is an authorized agent:

For the British Empire, except North America and Australasia:

Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, England. Prices of yearly subscriptions and of single copies may be had on application.

Business communications, remittances (in United States currency or its equivalent), and subscriptions should be addressed to THE UNIVERSITY OF CHICAGO PRESS, 5750 Ellis Avenue, Chicago 37, Illinois. All other communications should be addressed to Paul E. Steiner, M.D., University of Chicago, Chicago 37, Illinois.

Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

Entered as second-class matter, February 15, 1949, at the post office at Chicago, Ill., under the Act of March 3, 1879.

V

CANCER RESEARCH

VOLUME 9 JUNE 1949 NUMBER 6

Serum Proteins in Cancer*

CHARLES HUGGINS, M.D.

(*From the Department of Surgery, University of Chicago, Chicago 37, Illinois*)

Without doubt the greatest handicap to cancer research has been the small number of quantitative methods available for measuring the activity of tumors. Until the last decade, with a few exceptions, most notably in investigations with Warburg techniques, the science of cancer research was attempting to operate without precise measurements, a feat commendable in the attempt but clearly inefficient if not impossible. This accounts in large part, I think, for the slow rate of development of important facts about this difficult problem of growth.

Since 1929 it has been possible to recognize the presence, and more recently the activity of several neoplasms by chemical means. With the exception of myeloma, in all cases the component measured has represented an abnormally large concentration of a normal constituent of bodily cells. No specific neoplastic product as yet has been recognized quantitatively. Mostly the devices have concerned the tumors of man largely because chemical analysis of liquids is somewhat easier than solids and men are juicier than mice. The following components are available for measurement of cancer activity:

1. Bence-Jones proteinuria in myeloma.
2. Melanin precursors in the urine in melanoma; these colorless compounds after excretion can be oxidized (45) to melanin.
3. Chorionic gonadotrophin in the blood and urine (64, 16) in trophoblastic tumors of the testis and uterus.
4. Ketosteroids in the blood and urine of patients with tumors of the adrenal cortex (51, 13) and of the interstitial cells (57) of the testis. The androgenic hormones, *trans*-dehydroandrosterone (10) and androsterone (57) have been isolated in these cases in large amounts.

The most highly developed diagnostic agents in cancer are found among the proteins of plasma, the principal subject of this paper which, it is hoped, will supplement the recent reviews of Toennies (56) and Gutman (24).

There are fashions in science as in ladies apparel. After the work on proteins by Emil Fischer and his contemporaries early in this century, protein investigations were greatly influenced by the methods of the Swedish physical chemists which have yielded results of high importance. Recently there has been a return to the techniques of pure organic chemistry in the elucidation of the reactive groups of proteins.

ENZYMES

The overall activity of enzymes must be interpreted not only in terms of what amounts of these catalysts are present but also in the light of associated promoters and inhibitors of enzymic action.

(*a*) *Alkaline phosphatase.*—Kay (33, 34) made the important discovery that in hyperplastic disturbances of bone there are abnormally large amounts of alkaline phosphatase in the serum, the increases being correlated in a general way with the severity of the disease. In this connection increased osteoblastic activity leads to abnormally great fabrication of the enzyme which finds its way into the blood. Two effects occur in connection with skeletal tumors. Primary osteogenic tumors as such cause an elevation. Also, in connection with osteoblastic carcinomatous metastasis the reaction is due to the proliferation of non-malignant

* Presidential address delivered at the Fortieth Annual Meeting of the American Association for Cancer Research, Detroit, Michigan, April 16, 1949. The experiments done by the writer were supported by grants from Mr. Ben May, Mobile, Alabama, and from the American Cancer Society recommended by the Committee on Growth, the National Research Council.

osteoblasts which is evoked by the presence of certain epithelial tumors (*e.g.* prostate) in the skeleton; most metastatic tumors in bone however do not stimulate osteoblast growth. Control of the neoplasm is reflected *inter alia* in the movements of the osteoblastic mass set in motion by the regression of neoplasms and demonstrable by estimations of this enzyme. In most patients with skeletal metastasis from prostatic cancer, the induced remission (28) is followed by a temporary augmented stimulation of osteoblastic activity with increased elevation of alkaline phosphatase for some weeks, followed by a return of the enzyme level to normal. Clearly the reaction of the host tissue to the activity of a neoplasm may be followed closely by determining systematically the alkaline phosphatase of serum in patients with metastatic cancer resident in the skeleton.

(*b*) *Acid phosphatase.*—The discovery of Gutman and Gutman (22) that this enzyme is increased in the serum in prostatic cancer has added to the elegance of diagnosis and prognosis in this disease and also has provided a useful measuring device for investigative purposes. The nature of proof is sometimes of greater interest than that which is proven; by means of the acid and alkaline phosphatases of serum it was first demonstrated (28) that widespread carcinomatosis in man frequently is susceptible to more or less control through the use of chemotherapeutic agents. The control in a small percentage (20 per cent) of cases in the author's series of patients with prostatic cancer has lasted more than nine years and appears to be of indefinite duration.

Prostatic cancer no matter how large does not effect elevation of the phosphatases of serum, so long as the disease is confined to the locale of the pelvis.

The acid phosphatase content of serum rises only when the tumor has metastasized to bone marrow, lymph node, or liver. Apparently no mechanism exists for the entrance of molecules of so large a size as acid phosphatase except in regions where production of the plasma proteins normally takes place. While slight elevations of acid phosphatase in the serum occur in several non-malignant diseases, such as osteopetrosis, considerable elevations (*e.g.* values greater than 10 King and Armstrong units per 100 cubic centimeters) indicate metastatic carcinomatosis of the prostatic gland.

(*c*) *Aldolase (Zymohexase), and Isomerase.*—Aldolase is an enzyme widely distributed in animal tissue which catalyzes the reversible cleavage of fructose-1-6-diphosphate into the phosphates of glyceraldehyde and dihydroxyacetone, the equilibrium between the two products being established by a second enzyme triose-phosphate isomerase. Warburg and Christian (60) found that these enzymes were very much increased in the serum of rats bearing large Jensen sarcomas, the rise being roughly parallel to the size of the tumors; the enzymes in the serum of normal rats were not elevated. Sibley and Lehninger (50) using a simplified method of their own devising confirmed these findings with other tumors of rats; surgical excision of the tumor or the administration of ethyl carbamate caused a decline of aldolase to normal levels. Further, they found a significant elevation of aldolase in 20 per cent of 104 cases of cancer in man although there was no obvious correlation with the clinical findings.

Enzyme Inhibitors

(*a*) *Anti-proteolytic factors.*—Brieger and Trebing (9) demonstrated that serum from cancer patients could inhibit the proteolytic effects of pancreatic extracts. Using more refined methods Clark *et al.* (11) have reinvestigated this problem finding that the anti-trypsin factor of serum is greatly increased in cancer. They incubated dilutions of serum with trypsin, then added fibrinogen and later thrombin; the presence of a clot indicates undigested fibrin, hence the presence of an anti-trypsin factor and the greatest dilution of serum which permits coagulation is the end-point. Clark reported that 75 per cent of cancer patients gave positive reactions, 7 per cent were doubtful and 18 per cent negative; there were 9 per cent of false positive reactions in patients with advanced tuberculosis or active infections.

(*b*) *Tyrosinase inhibitor.*—Duboff and Hirshfeld (14) reported that a factor in the blood serum of cancer patients inhibited the aerobic oxidation of tyrosine by crude potato tyrosinase, suggesting that this inhibition might form the basis of a serum test for cancer. Marx (39) found that there was no close correlation between malignancy and tyrosinase inhibition although there was a small but statistically significant difference between normal and cancer sera.

(*c*) *Hyaluronidase inhibitor.*—Hakanson and Glick (25) reported that the inhibitor of hyaluronidase present in human serum was some 52 to 140 per cent increased in cancer; significant increases also occur in infections.

NON-ENZYMATIC PLASMA PROTEINS

In this field the greatest volume of work has been concerned with separation of proteins by electro-chemical or centrifugal methods. In the Tiselius (55) technique of electrophoresis a current

is passed under standard conditions through a solution containing a mixture of proteins such as plasma. Many proteins migrate at varying rates producing a series of boundaries which may be identified by optical and photographic means. The simplicity of the method is also its weakness in that the separation of proteins depends on the single property of differential migration in an electric field and certain proteins very different with respect to size and chemical composition have similar mobilities causing them to appear in a homogeneous peak.

The Svedberg method of ultra-centrifugation actually has had a more restricted usefulness than electrophoresis in the study of whole plasma because of more limited resolution unless many photographs are taken during examination of this complex mixture of proteins; its chief value lies in estimating the molecular size of purified proteins and in determining the homogeneity of prepared fractions. In these situations it is indispensable.

Total protein.—In most cases of significant cancer there is hypoproteinemia (48, 53) and all investigators report moderate to pronounced decreases in late cancer; the only known exception is myeloma where the total protein content of plasma is usually considerably increased. In this discussion myeloma will be considered in a separate category. Excluding myeloma, electrophoretic analysis gives evidence of wasting disease (41, 48) but is not specific for cancer.

Hypoproteinemia largely signifies hypoalbuminemia. In the classical consideration, hypoproteinemia implies deficiencies of intake, absorption or synthesis of protein or, on the other hand, increases of wear-and-tear (catabolism) or excretion. Any or all of these may be operative in cancer.

Fibrinogen.—Frequently but not invariably (17, 18, 53) the plasma fibrinogen is increased in cancer—at times doubled or quadrupled. Also, in cancer there is usually an increased sedimentation rate of the erythrocytes due at least in part to the increased fibrinogen. Gray and Mitchell (19) first added electrophoretically pure proteins to heparinized whole blood studying the rate of sedimentation thereafter; fibrinogen was more efficient in increasing the rate than any of the globulins of which γ-globulin was least effective while on the contrary albumin slowed the rate of settling. Following the excision of gastric cancers the fibrinogen and α-globulin (41) levels returned to normal in a few weeks.

Albumins.—The plasma albumin content is always decreased in the presence of a considerable cancer. Petermann and Hogness (41) found that in gastric cancer the level is far below the lowest normal; following gastrectomy, increases (41) in albumin are extremely slow even when the patient is in strongly positive nitrogen balance. It is the last component to return to normal after recovery (48).

Luetscher (37) showed that purified equine or human serum albumin although forming a single boundary at pH 7.4, separated into two components at pH 4 in acetate buffer; the faster moving component constituting two-thirds of the normal albumin, in common with most of the plasma proteins, carried a positive charge while the slower fragment moved to the anode. In cirrhosis and certain other diseases the faster moving component was diminished in amount leaving the negatively charged albumin preponderant. Petermann and Hogness (42) found that in gastric and pulmonary cancers and in some lymphatic leukemias the amount of the negatively charged protein component of plasma was significantly increased.

Globulins.—The α and β globulins are associated with much lipid and carbohydrate components (12) and the γ-globulins are, at least in part, immune bodies. Isolated α_2 globulin has a higher polysaccharide content (48) than any other plasma protein.

The α-fractions are often increased in chronic infection (49) and in conditions of increased tissue destruction irrespective of its cause. In cancer both of the α-globulins are commonly increased but the α_2 fraction to a greater extent than α_1, often conspicuously so. Seibert *et al.* (47) discovered an increased polysaccharide level in the serum always associated with increased α_2 in both cancer and advanced pulmonary tuberculosis. These elevations which may amount to as much as two-fold increases above the normal values are interpreted by them as concomitant upon tissue destruction; the polysaccharide level is not dependent on the state of metabolism (48).

The β and γ globulins are usually not very abnormal in cancer serum, although in certain cases with metastasis to the liver and jaundice (48) and also in Hodgkin's disease (43) the γ-fraction is slightly increased.

Multiple Myeloma.—In common with other neoplasms this disease when full blown is associated with decreased albumin (35, 23) and increased fibrinogen in the plasma but the most striking characteristic is an increased total plasma protein content. While the plasma proteins may be normal in amount and pattern (24, 36) in 62 per cent of 282 cases (23) the values were greater than 8 grams per cent; the total protein has been found as high as 13.8 grams per cent, three-quarters of the increment being "globulin" (40).

This rare disease has been much studied since Bence-Jones described the occurrence and striking thermal coagulative characteristics of the albumosuria in myeloma. These proteins are not globulins and have a molecular weight (54) of about 37,000. Using neutral salt precipitation techniques the increased protein of plasma has been salted-out in different cases with various sodium sulfate concentrations (23) between 13.5 and 21.5 per cent thus accompanying any of the globulin or albumin fractions; usually it is precipitated (23) with the 17.4 per cent fraction—the Pseudoglobulin I in the Howe terminology.

Bayne-Jones and Wilson (1) could demonstrate at least two antigenically different groups of urinary Bence-Jones proteins. Certain patients excrete a single protein of these types while others have both kinds in the urine (27).

The electrophoretic patterns of myeloma serum may be normal (24). More commonly there are increases of either the β (36) or γ (35) peaks, or an intermediary (M) spike (23) between them representing an abnormal pattern. These patterns are of great diagnostic importance provided other supporting data are present. By adding Bence-Jones proteins obtained from the urine of different patients to normal serum (23) it was possible to reproduce the several electrophoretic patterns found in clinical patients. Because of the lack of good methods for estimating Bence-Jones protein quantitatively in plasma it is difficult at the present time to differentiate between the increased protein fragments in myeloma as being globulins or Bence-Jones proteins migrating with the same electromobility.

REACTIVE GROUPS OF SERUM PROTEINS

Many rather quaint, if not weird, but apparently related and suggestive observations have been made which seem to indicate a systemic defect in cancer. Most of the techniques have one thing in common—they are rather unattractive to chemists, but many a stout heart beats under a rude cloak.

Kahn (32) observed that the serum of cancer patients is deficient in "Albumin A," the most soluble albumin fraction precipitated only with the stronger concentrations of neutral salts. In the original test, later refined (26), three drops of ear blood were collected on paper, dried and then soaked in 37.2 per cent ammonium sulfate, a concentration which, by definition, did not cause the precipitation of "Albumin A." The paper was then placed in boiling water where cloud formation in the hot water comprised a negative test indicating the presence of protein not salted-out, while opalescence-to-clear was positive. In addition to cancer the test was positive in pregnancy, starvation and cirrhosis.

The enzymes papain and methyl glyoxalase require -SH groups for activation (59). Purr and Russel (44) dialyzed papain to remove the -SH activator and then added blood from patients or rats with cancer; the whole blood in cancer had less activating power than normal blood. These findings were confirmed by Waldschmidt-Leitz *et al.* (58) and extended to partly inactivated glyoxalase; they observed that the activating groups of blood were resident in the serum, that of cancer being about one-half as effective in this regard as normal serum.

As shown by polarographic analysis also, sulfhydryl groups are deficient in cancer serum. Brdička (6) added an ammoniacal cobalt solution to serum and found that the catalytic polarographic waves were smaller in cancer than in normal serum. Using sera denatured in several ways the differences were more pronounced (7) being 3 to 50 per cent lower in cancer than the height obtained with normal serum; acute inflammation yielded results similar to cancer. On hydrolysis of sera with boiling 5*N* HCl, the hydrolysates (8) of cancer serum were found to contain less cystine than normal serum. It has long been known that there is in serum a small quantity of non-dialyzable proteose which is soluble in sulfosalicylic or trichloroacetic acids but is precipitated by more efficient protein precipitants such as phosphotungstic acid; these proteins are not heat coagulable. Brdička (8) made the important observation that after sulfosalicylic acid treatment with removal of the precipitated proteins the catalytic wave is higher (more proteoses) in cancer than in normal serum. Brdička's interpretation of these findings was that in cancer there is a pathologic *abbau* of the serum albumins. Neither of these findings is specific for cancer; they also occur in the serum in inflammatory states.

These indications of increased proteoses in cancer serum were confirmed by Winzler and Burk (61) using the blood of rats and rabbits with malignant tumors and inflammatory processes; the increase of proteoses above normal may be 12-fold and small quantities of the proteins added to culture media stop the growth of yeasts. Further studies by Winzler (62, 63) and associates revealed that the proteoses are mucoproteins; the carbohydrate/tyrosine ratio was the same in cancer as in normal serum demonstrating that the mucoproteins are similar in both states. The mucoprotein has an isoelectric point below that of serum

albumin and it contributes to the α-globulin fraction when electrophoresis is done at pH 8.4.

The reducing power of serum in cancer is decreased. Savignac, Gant and Sizer (46) added serum and alkali to methylene blue and boiled the mixture under standard conditions, observing the time required for decolorization. The reducing time of the dye is prolonged in cancer, uremia, and cirrhosis and they suggested that the methylene blue reduction test depended on the actual or potential reducing groups in the albumin fraction. Considering the mechanisms of the reduction of methylene blue by serum under the alkaline conditions (pH 11) employed by Savignac and coworkers, Stadie (52) demonstrated that heat converted a large part of the bound sulfur to S^{--}, which was the sole agent responsible for the reduction; he could find no correlation between the reduction time and the presence or absence of malignancy in 315 experiments.

Black (2) simplified the methylene blue reduction test and also omitted alkali from the system and confirming Savignac found a tendency towards deficient reduction in cancer serum which he ascribed to the albumin fraction. In the Black test methylene blue is added to serum and the tubes are placed in a bath of boiling water, the time of decolorization being determined. The plasma of patients (2) with malignant diseases "could be differentiated with a high degree of accuracy"; false positives occurred when the serum proteins were less than 5 grams per cent, and in cirrhosis. Black (3, 4) postulated that the decreased reduction of methylene blue by cancer serum might be due to changes of spatial configuration of the albumin molecule accounting for a delayed appearance (unmasking) of the reducing groups, there being no significance in the total number potentially present.

In a small series of cases we also found in this laboratory decreased reducing properties of the blood in cancer. Jensen *et al.* (31) examined the reducing power of serum by measuring colorimetrically the amount of triphenylformazan (TPF) formed when serum was heated with triphenyltetrazolium chloride at 80° C. Sera from 108 individuals were compared on the basis of their reducing power per milligram of serum protein. The results were expressed in the form of an arbitrary index,

$$\frac{\text{micrograms TPF in 0.5 ml.}}{\text{gm. of protein per cent}}$$

In a group of 60 patients with various types of cancer 85 per cent showed a low reducing index of 9.9 or below; of the 15 per cent whose index lay above this figure two-thirds were cases of prostatic carcinoma which appeared clinically to be under good control by anti-androgenic measures. In a group of 48 individuals having no disease or nonmalignant pathology, 80 per cent showed a reducing index above 10.0.

Abnormalities of reactive groups are also demonstrable by the heat coagulation of cancer serum. Black *et al.* (5) found that the plasma of cancer patients coagulated to a greater extent than normal plasma, the finding apparently being due to the increased level of fibrinogen since it was not operative when serum was used. In this test the density of diluted serum was determined in a colorimeter before and after heating for 10 seconds.

In contrast to plasma, cancer serum is deficient in its thermal coagulation properties. Some qualitative observations (15) demonstrated that with respect to normal serum, less flocculation occurred in heated serum from cancer patients. These observations have been recently confirmed (30) and placed on a quantitative basis.

Boiling undiluted serum for 30 minutes always causes it to coagulate while on the other hand any serum can be diluted to a point where it will not solidify when heated; normal serum may be diluted to a greater extent than cancer serum and still retain its capacity to gel. The serum from 84 normal persons coagulated when diluted so that the total protein was less than 1.5 grams per cent; only 8 per cent of cancer sera coagulated in so great dilution. Determination of the least coagulable protein concentration is a simple though rough screen for cancer.

The deficient coagulative ability of cancer serum may be demonstrated in a more refined manner by measuring the inhibition of thermal coagulation by iodoacetate. Gel formation in protein solutions is believed to depend on the formation of a 3-dimensional network of polypeptide chains. Sulfhydryl compounds (*e.g.* cysteine, dimercaptopropanol) greatly enhance thermal coagulation while iodoacetate (29) in appropriate and small amounts blocks it. This effect of iodoacetate is unique among a class of iodinated compounds since iodoacetamide, methyl iodoacetate and iodoacetone do not inhibit coagulation. It has been shown by a number of investigators that iodoacetate and iodoacetamide react with -SH groups and to a less extent with amino groups of proteins; no appreciable difference exists at 100° C. in the rate of reaction of the model compound cysteine with iodoacetate or iodoacetamide. Both ends of the iodoacetate ion therefore are clearly involved in its peculiar inhibition of the coagulation of proteins by heat; iodoacetate combining with reactive

groups of proteins increases the net negative charge on the protein molecule by adding mutually repelling electro-negative groups which prevent the close apposition of adjacent molecules.

Smaller quantities of iodoacetate are required to block the thermal coagulation of cancer serum (30) than normal serum. By determining the smallest quantity of iodoacetate required to prevent coagulation of serum by heat and relating this to the protein content of serum it was found that all of 85 consecutive cancers fell in a group with a low iodoacetate index (less than 9). The serum of all patients with active pulmonary tuberculosis resembled cancerous serum while that of pregnant women and newborn infants reacted like normal adult serum.

Madden and Whipple (38) have recently reviewed the evidence that serum albumin is made in large part in the liver. Griffin and Baumann (21) demonstrated that homogenates of livers of rats fed *m'*-methyl-*p*-dimethylaminoazobenzene failed to coagulate at 100° C. whereas similar homogenates from normal rats coagulated completely in 1 to 5 minutes. Greenstein and Andervont (20) in their famous transplantation experiments involving the growth of tumors in the tails of mice found that the catalase content of the liver which of course was not directly invaded by the tumor was profoundly depressed; on caudectomy, thereby removing the neoplasm, the hepatic catalase content gradually rose to its normal level. Through these experiments Greenstein demonstrated clearly that the presence of a tumor even in one of the extremities of the host depressed the synthesis of an enzyme in the liver.

In summary the following evidence implies a defect in the serum albumin in cancer: a quantitative deficiency in one of the albumin fractions ("Albumin A," positively charged albumin); decreased cystine as indicated by decreased sulfur catalytic waves; decreased reducing groups; deficient thermal coagulation. The defect is not specific for cancer, occurring also in infection. The mechanism of the production of the albumin abnormality is unknown but presumably is due to the effect of some chemical agent on hepatic synthesis.

REFERENCES

1. Bayne-Jones, S., and Wilson, D. W. Immunological Reactions of Bence-Jones Proteins. II. Differences Between Bence-Jones Proteins from Various Sources. Bull. Johns Hopkins Hosp., **33**:119–125, 1922.
2. Black, M. M. Changes in the Reducing Power of Serum or Plasma of Patients with Malignant Neoplastic Disease. Cancer Research, **7**:321–325, 1947.
3. Black, M. M. Sulfhydryl Reduction of Methylene Blue with Reference to Alterations in Malignant Neoplastic Disease. Cancer Research, **7**:592–594, 1947.
4. Black, M. M. Plasma Reduction of Methylene Blue. Science, **108**:540, 1948.
5. Black, M. M., Kleiner, I. S., and Bolker, H. Changes in the Heat Coagulation of Plasma from Cancer Patients. Cancer Research, **8**:79–82, 1948.
6. Brdička, R. Application of Polarographic Effect of Proteins in Cancer Diagnosis. Nature, **139**:330, 1937.
7. Brdička, R. Polarographic Investigations in Serological Cancer Diagnosis. Nature, **139**:1020–1021, 1937.
8. Brdička, R. Serologische Untersuchungen mit Hilfe der polarigraphischen Methode und ihre Bedeutung für die krebs Diagnostik. Acta Union Internat. Contra Cancer, **3**:13–30, 1938.
9. Brieger, L., and Trebing, J. Ueber die antitryptische Kraft des menschlichen Blutserums, inbesondere bei Krebskranken. Berl. Klin. Wchnschr., **54**:1041–1044, 1908.
10. Callow, R. K. Isolation of the Male Hormone Present in the Urine of a Patient with an Adrenal Tumor. J. Soc. Chem. Ind., **55**:1030, 1936.
11. Clark, D. G. C., Cliffton, E. E., and Newton, B. L. Antiproteolytic Activity of Human Serum with Particular Reference to its Changes in the Presence and Considerations of its Use for Detection of Malignant Neoplasia. Proc. Soc. Exper. Biol. and Med., **69**:276–279, 1948.
12. Cohn, E. J., Oncley, J. L., Strong, L. E., Hughes, W. L., Jr., and Armstrong, S. H., Jr. Preparation and Properties of Serum and Plasma Proteins. IV. A System for the Separation into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids. J. Am. Chem. Soc., **68**:459–475, 1946.
13. Dorfman, R. I. The Hormones: Physiology, Chemistry and Applications, edited by G. Pincus and K. V. Thimann. Vol. 1, p. 513. New York: Academic Press, Inc., 1948.
14. Duboff, G., and Hirshfeld, S. Demonstration of an Enzyme-inhibiting Factor in the Serum of Cancer Patients (a preliminary study). Cancer Research, **6**:57–60, 1946.
15. Ehrentheil, O., and Weis-Ostborn, W. Ueber das verschiedene Verhalten des erhitzten Serums bei Carcinom und bei Anaemia Perniciosa. Klin. Wchnschr., **2**:585–586, 1923.
16. Ferguson, R. S. Quantitative Behavior of Prolan A in Teratoma Testis. Am. J. Cancer, **18**:269–295, 1933.
17. Goldfeder, A. Studien an chemischen Bestandteilen im Blute von Krebs-kranken. Ztschr. f. Krebsforsch., **40**:394–409, 1934.
18. Gram, H. C. The Results of a New Method for Determining the Fibrin-percentage in Blood and Plasma. Acta med. Scandinav., **56**:107–162, 1922.
19. Gray, S. J., and Mitchell, E. B. Effect of Purified Protein Fractions on Sedimentation Rate of Erythrocytes. Proc. Soc. Exper. Biol. and Med., **51**:403–404, 1942.
20. Greenstein, J. P., and Andervont, H. P. The Liver Catalase Activity of Tumor Bearing Mice and the Effect of Spontaneous Regression and of Removal of Certain Tumors. J. Nat. Cancer Inst., **2**:345–355, 1942.
21. Griffin, A. C., and Baumann, C. A. Note on the Heat Coagulation of Homogenates from Normal and Precancerous Livers. Cancer Research, **8**:135–138, 1948.
22. Gutman, A. B., and Gutman, E. B. An "Acid" Phosphatase Occurring in the Serum of Patients with Metastasizing Carcinoma of the Prostate Gland. J. Clin. Investigation, **17**:473–478, 1938.
23. Gutman, A. B., Moore, D. H., Gutman, E. B., McClellan, V., and Kabat, E. A. Fractionation of Serum

Proteins in Hyperproteinemia with Special Reference to Multiple Myeloma. J. Clin. Investigation, **20**:765–783, 1941.

24. GUTMAN, A. B. The Plasma Proteins in Disease. Advances in Protein Chemistry. Vol. IV, pp. 155–250. New York: Academic Press, Inc., 1948.
25. HAKANSON, E. Y., and GLICK, D. Mucolytic Enzyme Systems. III. Inhibition of Hyaluronidase by Serum in Human Cancer. J. Nat. Cancer Inst., **9**:129–132, 1948.
26. HANKE, M. E., KAHN, H., and FELSHER, R. Substances which Decrease the Solubility of Albumin S in Human Cancer. Federation Proceedings, **3**:58, 1944.
27. HEKTOEN, L., and WELKER, W. H. Immunological Differences of Crystalline Bence-Jones Proteins. Biochem. J., **34**:487–489, 1940.
28. HUGGINS, C., and HODGES, C. V. Studies on Prostatic Cancer. I. The Effect of Castration, of Estrogen and of Androgen Injection on Serum Phosphatases in Metastatic Carcinoma of the Prostate. Cancer Research, **1**:293–297, 1941.
29. HUGGINS, C., and JENSEN, E. V. Thermal Coagulation of Serum Proteins. I. The Effects of Sulfhydryl Groups and of Iodoacetate and Iodoacetamide on Coagulation. J. Biol. Chem., 1949, In press.
30. HUGGINS, C., MILLER, G. M., and JENSEN, E. V. Thermal Coagulation of Serum Proteins. II. Deficient Coagulation in Cancer and the Iodoacetate Index. Cancer Research, **9**:177–182, 1949.
31. JENSEN, E. V., CAMARATA, P., and HUGGINS, C. Unpublished observations.
32. KAHN, H. Die Chemie der malignen Tumoren und die chemischen Veränderungen im krebskranken Organismus. Ergebn. d. inn. Med. u. Kinderh., **27**:365–422, 1925.
33. KAY, H. D. Plasma Phosphatase in Osteitis Deformans and in Other Diseases of Bone. Brit. J. Exper. Path., **10**: 253–256, 1929.
34. KAY, H. D. Plasma Phosphatase. II. The Enzyme in Disease Particularly in Bone Disease. J. Biol. Chem., **89**: 249–266, 1930.
35. KEKWICK, R. A. The Serum Proteins in Multiple Myelomatosis. Biochem. J., **34**:1248–1257, 1940.
36. LONGSWORTH, L. G., SHEDLOVSKY, T., and MACINNES, D. A. Electrophoretic Patterns of Normal and Pathological Blood Serum and Plasma. J. Exper. Med., **70**:399–413, 1939.
37. LUETSCHER, J. A. Serum Albumin. II. Identification of More than One Albumin in Horse and Human Serum by Electrophoretic Mobility in Acid Solution. J. Am. Chem. Soc., **61**:2888–2890, 1939.
38. MADDEN, S. C., and WHIPPLE, G. H. Plasma Proteins: Their Source, Production and Utilization. Physiol. Rev., **20**:194–217, 1940.
39. MARX, W. The Influence of Normal and Cancer Blood on Tyrosinase Activity *in vitro*. Cancer Research, **9**:114–120, 1949.
40. PERLZWEIG, W. A., DELRUE, G., and GESCHICKTER, C. Hyperproteinemia Associated with Multiple Myelomas. Report of an Unusual Case. J.A.M.A., **90**:755–757, 1928.
41. PETERMANN, M. L., and HOGNESS, K. R. Electrophoretic Studies on the Plasma Proteins of Patients with Neoplastic Disease. I. Gastric Cancer. Cancer, **1**:100–103, 1948.
42. PETERMANN, M. L., and HOGNESS, K. R. Electrophoretic Studies on the Plasma Proteins of Patients with Neoplastic Disease. II. An Acid Protein in the Plasma. Cancer, **1**:104–108, 1948.
43. PETERMANN, M. L., KARNOXSKY, D. A., and HOGNESS, K. R. Electrophoretic Studies on the Plasma Proteins of Patients with Neoplastic Disease. III. Lymphomas and Leukemia. Cancer, **1**:109–119, 1948.
44. PURR, A., and RUSSEL, M. Die Aktivierungsfähigkeit des Papains, angewendet auf eine Bestimmungsmethode physiologisch aktiver Stoffe in Blut. Ztschr. f. physiol. Chem., **228**:198–206, 1934.
45. ROTHMAN, S. Studies on Melanuria. J. Lab. and Clin. Med., **27**:687–692, 1942.
46. SAVIGNAC, R. J., GANT, J. C., and SIZER, I. W. Reducing Properties of Serum from Malignant and Nonmalignant Patients and from Normal Individuals. A.A.A.S. Research Conference on Cancer, 241–252, 1945.
47. SEIBERT, F. B., PFAFF, M. L., and SEIBERT, M. V. A Serum Polysaccharide in Tuberculosis and Carcinoma. Arch. Biochem., **18**:279–295, 1948.
48. SEIBERT, F. B., SEIBERT, M. W., ATNO, A. J., and CAMPBELL, H. J. Variation in Protein and Polysaccharide Content in the Chronic Diseases, Tuberculosis, Sarcoidosis and Carcinoma. J. Clin. Investigation, **26**:90–102, 1947.
49. SHEDLOVSKY, T., and SCUDDER, J. A. Comparison of Erythrocyte Sedimentation Rates and Electrophoretic Patterns of Normal and Pathological Human Blood. J. Exper. Med., **75**:119–126, 1942.
50. SIBLEY, J. A., and LEHNINGER, A. L. Aldolase in the Serum and Tissues of Tumor Bearing Animals. J. Nat. Cancer Inst., **9**:303–309, 1949.
51. SLOT, W. J. B. The Relation of Sex Hormones in a Case of Virilism by Hypernephroma. Acta med. Scandinav., **89**: 371–376, 1936.
52. STADIE, W. C. The Reducing Properties of Serum from Patients with Malignant Disease. Science, **108**:211, 1948.
53. STARLINGER, W., and WINANDS, E. Ueber das Verteilungsverhältnis der zirkulierenden Eiweisskörper im Verlaufe krankhafter Zustände. Ztschr. f. d. ges. exper. Med., **60**: 185–207, 1928.
54. SVEDBERG, T., and PEDERSEN, K. O. The Ultracentrifuge. Oxford: Clarendon Press, 1940.
55. TISELIUS, A. Electrophoretic Analysis of Normal and Immune Sera. Biochem. J., **31**:1464–1477, 1937.
56. TOENNIES, G. Protein-Chemical Aspects of Cancer. Cancer Research, **1**:193–229, 1947.
57. VENNING, E. H. Étude hormonale sur un cas de tumeur interstitielle du testicule. Rev. canad. de biol., **1**:571–572, 1942.
58. WALDSCHMIDT-LEITZ, E., CONRATH, O., and GLOEDITSCH, J. Versuche zur Diagnostik bösartiger Geschwülste. Naturwissen, **4**:60, 1937.
59. WALDSCHMIDT-LEITZ, E., and PURR, A. Ueber Zookinase. Ztschr. f. physiol. Chem., **198**:260–266, 1931.
60. WARBURG, O., and CHRISTIAN, W. Garungsfermente im Blutserum von Tumor-ratten. Biochem. Ztschr., **314**: 399–408, 1943.
61. WINZLER, R. J., and BURK, D. Blood Proteose and Cancer. J. Nat. Cancer Inst., **4**:417–428, 1944.
62. WINZLER, R. J., DEVOR, A. W., MEHL, J. W., and SMYTH, I. M. Studies on the Mucoproteins of Human Plasma. I. Determination and Isolation. J. Clin. Investigation, **27**: 609–616, 1948.
63. WINZLER, R. J., and SMYTH, I. M. Studies on the Mucoproteins of Human Plasma. II. Plasma Mucoprotein Levels in Cancer Patients. J. Clin. Investigation, **27**: 617–619, 1948.
64. ZONDEK, B. Ueber die Hormone des Hypophysenvorderlappens. III. Follikelreifungshormon (Prolan A) und Tumoren. Klin. Wchnschr., **9**:679–682, 1930.

The Mammary Tumor Milk Agent Given to Adult Female Mice Following Splenectomy and Vital Staining*

PAULO R. F. BORGES

(From the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine)

Studies on the mammary tumor milk agent of the mouse (14) would be facilitated by the development of a rapid test for its detection. The usual method of testing for the presence of this agent requires that test animals be observed for a long period of time before an estimate can be made of the percentage of mammary tumors obtained, as the latent period of the milk agent runs from 6 to 24 months (3).

By the use of complement-fixation tests Bennison (8) obtained results showing that spleen extracts from mice possessing the mammary tumor milk agent fix more complement than spleen extracts from agent-free mice. Recently Imagawa, Green, and Halvorson (20) reported a precipitin test for mouse tissues containing the milk agent. Results from the use of such time-saving tests in routine work are eagerly awaited.

Experiments have shown that mice of susceptible strains become more resistant to the milk agent with increasing age (4, 5). Adult mice given the milk agent develop few mammary tumors (2, 10, 13). However, Dmochowski (17) succeeded in inducing a high incidence of breast tumors by injecting repeated doses of dried breast tumor tissue into 4 months old hybrid female mice which were genetically susceptible to mammary tumors, but which lacked the milk agent.

Considering that the role played by the reticuloendothelial system in the resistance to the growth of tumors remains unsettled (21), the following experiments were undertaken to ascertain whether it would be possible to hasten the action of the milk agent in old mice submitted to splenectomy and vital staining, hoping to develop a more rapid bioassay technique for the milk agent.[1]

MATERIALS AND METHODS

Hybrid BDF_1 (C57 Black ♀ × dba ♂) and BAF_1 (C57 Black ♀ × A ♂) mice were used as test animals. The age of the mice at the beginning of the experiments ranged from 10 to 15 months. All were breeding females, born and raised in the Roscoe B. Jackson Memorial Laboratory. The animals were kept on a diet of Purina Fox Chow pellets with an unlimited supply of water.

BDF_1 and BAF_1 hybrids are genetically susceptible to mammary tumors (9, 12, 24), but as they do not obtain the milk agent while being nursed by their low-cancer strain C57 Black mothers, they show a low incidence of mammary tumors under normal conditions. However, BDF_1 and BAF_1 are suitable as test animals as they show an increased percentage of breast tumors when supplied with the milk agent (9, 11, 17, 23).

Splenectomy was performed under nembutal anesthesia.

Sterile 0.5 per cent aqueous solution of trypan blue or 1 per cent aqueous solution of congo red was used in each of two groups of experiments. The injections of the dyes were given intraperitoneally, and started the following day after splenectomy was performed. Mice of the trypan blue group received injections for 2 months. In the beginning 0.2 cc. to 0.5 cc. of the dye was given at 3 to 7 day intervals until a definite blue color of the skin was shown. Later, doses of 0.2 cc. given at 4-day intervals were maintained until the end of the experiment. Mice of the congo red group were injected daily with 0.1 cc. to 0.3 cc. of the dye during the 4 days following splenectomy. From then on 0.3 cc. of congo red was injected every other day, occasionally at 3-day intervals, for 57 days.

Freshly excised spontaneous mammary tumors from C3H female mice were used as a source of the milk agent. Minced mammary tumors were suspended in 0.9 per cent sodium chloride solution in the proportion of 1 gm. of tumor tissue in 30 cc. of sodium chloride solution. The suspensions were cleared by centrifugation at 2000 r.p.m. for 10 minutes. The supernatant fluid was first passed through a Seitz filter, and then through a porcelain bacteria-tight "Selas" candle No. 02. Each mouse was injected intraperitoneally with 0.5 cc. of mam-

* This investigation was aided by a Finney-Howell Research Foundation Fellowship.

[1] These experiments were interrupted and partially ruined by the Bar Harbor fire in October, 1947.

mary tumor filtrate at 4 to 13 day intervals for the trypan blue group, and weekly for the congo red group. The injections of the filtrate were given on days not coinciding with the administration of either trypan blue or congo red.

Mice subjected to splenectomy and injected with trypan blue or congo red (but no milk agent), and non-splenectomized mice given the milk agent alone (single or repeated doses) were run as controls.

The animals were kept under observation for 8 to 26 weeks until the experiments were accidentally interrupted. The results of these experiments, which were negative, are shown in Table 1.

TABLE 1

ATTEMPTS TO INDUCE MAMMARY TUMORS IN BREEDING FEMALE MICE BY INTRAPERITONEAL ADMINISTRATION OF THE MAMMARY TUMOR MILK AGENT FOLLOWING SPLENECTOMY AND VITAL STAINING

Group	Stock	Age at the beginning of the experiments *Months*	Number of mice	Treatment*	Number of injections: Trypan blue†	Congo red‡	Milk agent§	Time under observation *Weeks*	Average age of mice at death *Months*	Results
Experimental	BDF_1	13–15	7	Sp.-TB-MA	15		6	18	17.5	Negative
"	BAF_1	10–14	7	"	15		5	18	15.1	"
Control	"	10–14	9	Sp.-TB	15			17	15.3	"
"	"	10–14	9	MA			5	17	15.4	"
"	"	11	9	"			1	26	17.0	"
Experimental	"	11–14	8	Sp.-CR-MA		27	8	8	15.3	"
Control	"	11–14	6	Sp.-CR		27		8	15.5	"

* Sp., Splenectomy; TB, Trypan blue; MA, Milk agent; CR, Congo red.

† 0.5 per cent aqueous solution; 0.2 cc. to 0.5 cc. intraperitoneally.

‡ 1 per cent aqueous solution; 0.1 cc. to 0.3 cc. intraperitoneally.

§ Cell-free filtrate of spontaneous mammary tumor from C3H mice (1 gm. of tumor tissue in 30 cc. of 0.9 per cent sodium chloride solution); 0.5 cc. of filtrate intraperitoneally.

DISCUSSION

The purpose of the experiments reported here was to investigate whether it would be possible to hasten the appearance of mammary tumors in genetically susceptible hybrid mice, and thus work out a more rapid method of testing for the presence of the mammary tumor milk agent.

By injecting 4 months old BAF_1 breeding females with 1.5 gm. of dried breast tumor tissue, divided in 12 doses, Dmochowski (17) succeeded in obtaining a high percentage of mammary tumors after 13 to 26 months. The relative resistance of adult mice to the milk agent is thus believed to have been overcome by large doses of the agent.

The opinions about the role played by the reticulo-endothelial system in the resistance to tumor growth are discordant (7, 15, 16, 18, 19, 22, 25, 26). However, a great number of experiments on this subject seems to indicate that a decrease in the number of active reticulo-endothelial elements lowers the resistance to the growth of tumors. The contradictory results thus far obtained can be explained by the difference of materials and methods used by individual investigators, and by the great capacity of the reticulo-endothelial system to regenerate and to perform vicarious functions. This capacity is so pronounced that it cannot be decided to what extent procedures intended to produce a blockade will in fact determine a stimulation of the reticulo-endothelial system. Aschoff (6) himself recognized that it is difficult, and even impossible, to suppress altogether and permanently the functions of the reticulo-endothelial system. Yet it has been demonstrated that vital staining lowers or suppresses the resistance of animals to transplanted tumors (1, 27).

There are some possible factors to account for the negative results obtained in the present experiments: 1) The mice might have attained an age when the milk agent is no longer effective; 2) The dose of milk agent administered might not have been large enough to overcome their resistance; 3) The time during which the animals were kept under observation might not have been long enough for mammary tumors to develop; 4) The presence of trypan blue or congo red in the mouse might have interfered with the specific activity of the milk agent.

The very purpose of the present experiments explains why these factors were not obviated, and why mice which had been observed for 8 weeks were also included in the table.

SUMMARY

Attempts were made to develop a more rapid method of testing for the presence of the mouse mammary tumor milk agent.

Repeated doses of filtrates of freshly excised spontaneous C3H mammary tumors failed to induce mammary tumors in 10 to 15 months old BDF_1 and BAF_1 breeding females. The mice which previously had been submitted to splenectomy and

vital staining with trypan blue or congo red were kept under observation for 8 to 18 weeks, until the experiments were accidentally interrupted.

ACKNOWLEDGMENTS

The author is indebted to Dr. L. W. Law for suggesting these experiments, and to Mr. Sidney Chason for his assistance.

REFERENCES

1. Andervont, H. B. Studies on Immunity Induced by Mouse Sarcoma 180. Pub. Health Rep., **47**:1859–1877, 1932.
2. Andervont, H. B. Susceptibility of Young and of Adult Mice to the Mammary-tumor Agent. J. Nat. Cancer Inst., **5**:397–401, 1945.
3. Andervont, H. B. The Milk Influence in the Genesis of Mammary Tumors. A.A.A.S. Publ. No. 22 on Mammary Tumors in Mice, pp. 123–139, 1945.
4. Andervont, H. B., with assist. of McEleney, W. J. Effect of Ingestion of Strain C3H Milk in the Production of Mammary Tumors in Strain C3H Mice of Different Ages. J. Nat. Cancer Inst., **2**:13–16, 1941.
5. Andervont, H. B., Shimkin, M. B., and Bryan, W. R. Technique Suitable for Quantitative Studies on the Mammary Tumor Inciter of Mice. J. Nat. Cancer Inst., **3**:309–318, 1942.
6. Aschoff, L. Das reticulo-endotheliale System. Ergebn. d. inn. Med. u. Kinderh., **26**:1–118, 1924.
7. Baroni, V., and Comsia, O. Blocage du système réticulo-endothélial par l'encre de Chine et immunité envers le sarcome de Jensen. Compt. rend. Soc. de biol., **111**:1108–1111, 1932.
8. Bennison, B. E. Serologic Studies on Mouse Mammary Tumors. Program abstracts, Fourth Intern. Cancer Research Congress, St. Louis, Mo., U.S.A., 1947.
9. Bittner, J. J. Breast Cancer in Mice as Influenced by Nursing. J. Nat. Cancer Inst., **1**:155–168, 1940.
10. Bittner, J. J. Inciting Influences in the Etiology of Mammary Cancer in Mice. A.A.A.S. Research Conference on Cancer, pp. 63–95, 1945.
11. Bittner, J. J. Observations on the Inherited Susceptibility to Spontaneous Mammary Cancer in Mice. Cancer Research, **4**:159–167, 1944.
12. Bittner, J. J. Possible Method of Transmission of Susceptibility to Breast Cancer in Mice. Am. J. Cancer, **39**:104–113, 1940.
13. Bittner, J. J. Possible Relationship of the Estrogenic Hormones, Genetic Susceptibility, and Milk Influence in the Production of Mammary Cancer in Mice. Cancer Research, **2**:710–721, 1942.
14. Bittner, J. J. The Mammary Tumor Milk Agent. Ann. New York Acad. Sc., **49**:69–73, 1947.
15. Brüda, B. E. Über erfolgreiche Züchtung von Mäusetumoren auf entmilzten Ratten. Klin. Wchnschr., **7**:27, 1928.
16. Büngeler, W. Geschwulsttransplantation am gespeicherten Tier. Frankf. Zeitschr. f. Path., **35**:67–82, 1927.
17. Dmochowski, L. Age and Dosage in the Induction of Breast Cancer in Mice by the Mouse Mammary Tumour Agent. Brit. J. Exper. Path., **26**:192–197, 1945.
18. Dobrovolskaïa-Zavadskaïa, N., and Samssonow, N. L'influence de la splénectomie sur les greffes du cancer expérimental. Compt. rend. Soc. de Biol., **92**:1222–1224, 1925.
19. Erdmann, R. Erzeugung des Flexner-Jobling-Tumors durch Filtrate. Ztschr. f. Krebsforsch., **27**:69–82, 1928.
20. Imagawa, D. T., Green, R. G. (Deceased), and Halvorson, H. O. A Precipitin Test for Antigens Present in Mouse Tissues Containing the Milk Agent. Proc. Soc. Exper. Biol. and Med., **68**:162–166, 1948.
21. Jaffé, R. H. The Reticulo-endothelial System in Immunity. Physiol. Rev., **11**:227–327, 1931.
22. Munck, W. Über den Einfluss von Vital-Trypanblauspeicherung auf das Wachstum von verimpften Mäusecarcinom und -sarkom. (Verläufige Mitteilung). Ztschr. f. Krebsforsch., **26**:377–385, 1928.
23. Murray, W. S. Studies on the Effect of Foster Nursing and Its Relation to the Development of Mammary Carcinoma in the Mouse. Cancer Research, **1**:790–792, 1941.
24. Murray, W. S., and Little, C. C. Chromosomal and Extrachromosomal Influence in Relation to the Incidence of Mammary Tumors in Mice. Am. J. Cancer, **37**:536–552, 1939.
25. Oser, E. G., and Pribram, E. E. Ueber die Bedeutung der Milz in dem an malignem Tumor erkrankten Organismus und die Beeinflussung von Tumoren durch Milzbrei. Ztschr. f. exper. Path. u. Therap., **12**:295–302, 1913.
26. Psaromitas, D. Zur Frage des Wachstums artfremder Tumoren bei gespeicherten Tieren. Ztschr. f. Krebsforsch., **30**:77–105, 1930.
27. Saphir, O., and Appel, M. Attempts to Abrogate Immunity to the Brown-Pearce Carcinoma. Cancer Research, **3**:767–769, 1943.

Hypervolemia and Associated Changes in Mice Bearing a Transplanted Granulosa Cell Tumor*

E. E. Cliffton, M.D.,† and J. T. Wolstenholme, M.D.‡

(From the Departments of Surgery and Anatomy, Yale University School of Medicine, New Haven 11, Connecticut)

Mice bearing a transplanted granulosa cell tumor have an increased blood volume associated with sinusoidal dilatation in the liver, spleen, and adrenal glands. These changes were first described by Furth, Boon, and Sobel (3, 4, 5, 6). Growth of transplanted granulosa cell tumors has been obtained in a large percentage of castrated mice of the C57 strain[1] that received injections of a crude compound[2] (2). In connection with other studies, it was noted that a much larger quantity of heart blood was obtained from these animals than had been obtained previously from mice of a similar size of this or other strains either with or without tumors. At post-mortem an increase in the size of the liver and spleen was noted in these animals as compared to normal mice and mice bearing other tumors.

METHODS

A total of 116 mice of the C57 strain were used. The granulosa cell tumor (18 C57) was induced in an animal of the C57 strain by transplantation of an ovary into the spleen. The animals were castrated at 60 to 90 days of age and a small fragment of the tumor transplanted subcutaneously immediately following castration. The tumor transplants became palpable between 10 and 35 days after transplantation and attained a size of 1 × 1 cm. in 40 to 65 days. Early in the experiment the animals were sacrificed only when death seemed imminent; later in the experiment other animals were sacrificed with tumors of different sizes, the smallest being 6 × 6 mm. Sixty-two animals were treated with the crude compound diluted 4 parts to 1 part of ethanol. These animals received 0.05 cc. of the diluted crude compound 2 times weekly. Sixteen animals received 0.05 cc. of estradiol benzoate (25 μg in 0.05 cc. sesame oil) 2 times weekly. Ten animals received progesterone,[3] containing 5 mg. progesterone/cc., 0.05 cc. daily, and 5 animals were treated with 0.05 cc. sesame oil diluted with ethanol (4 parts of sesame oil to 1 part of ethanol) 2 times weekly. Twenty-three animals received no injections. All animals were kept on a standard diet and water *ad libitum*.

OBSERVATIONS

The animals with and without tumor growth gained weight progressively. The animals with granulosa cell tumors increased in weight more rapidly than the controls when the tumor attained an approximate diameter of 1 cm. The maximum gain in weight of tumor bearing animals, after subtraction of the tumor weight determined at necropsy, ranged from 3.3 to 13.6 gms.; 27 of 33 animals gained over 5.0 gms. and 7 animals gained over 10.0 gms. Animals without tumors gained from 0.0 to 9.7 gms.; only 7 of the 35 animals gained over 5.0 gms. The average increase in weight, less tumor weight, of the tumor bearing animals was 7.1 gms., while that of the animals without tumors was 3.0 gms.

The differences of weight increment were greater in animals that were mature at the beginning of the experiment. The animals without tumors that gained more than 5.0 gms. were immature at the start of the experiment; they weighed from 13.2 to 17.0 gms. Because the animals without tumors lived an average of 3 months longer than the tumor bearing animals before being sacrificed, they should have increased relatively more in weight.

The tumors were removed from 3 animals and in each instance the animal's weight returned to and remained at the level of the control animals. A local recurrence of the tumor developed 2 months later in one animal that subsequently in-

* This investigation was supported by a research grant from the U.S. Public Health Service and a fellowship grant from the American Cancer Society.

† Senior Research Fellow, American Cancer Society.

‡ Post-doctorate Research Fellow, National Institute of Health.

[1] Original mice obtained from L. C. Strong.

[2] Crude compound containing approximately 200–250 I.U. progesterone, obtained from the Glidden Company through the courtesy of A. E. Engstrom.

[3] Proluton obtained through the courtesy of Shering Corporation, Bloomfield, N.J.

creased in weight in a manner similar to the other tumor bearing animals.

The blood, obtained by cardiac puncture from tumor bearing animals, weighed from 1.0 to 3.3 gms.; from animals without tumors the blood weighed from 0.4 to 1.3 gms. The average weight of blood from tumor bearing animals was 2.1 gms. and from animals without tumors was 0.6 gms. (Chart I). At necropsy an increase in the size of the heart was noted without a concomitant increase in its weight, indicating that the increase in size was due to dilatation.

The livers of the animals with tumors showed marked vascular congestion; their surfaces presented an unusual mottled appearance. The weights of the livers in 51 tumor bearing animals ranged from 1.3 to 6.0 gms., an average weight of 2.5 gms. The livers of 30 animals without tumors weighed from 1.0 to 2.3 gms., an average weight of 1.5 gms. (Chart II).

Microscopic examination of the livers from animals with tumors revealed many dilated blood sinuses. All stages were noted from slight dilatation of the blood sinuses (Fig. 1) to marked dilatation of all blood channels and the formation of new blood-filled sinuses, in many instances isolating single cords of liver cells in pools of blood (Fig. 2). Necrosis of liver cells was not noted except in livers with extreme sinusoidal dilatation. The number of macrophages was increased.

The spleens of the 51 tumor bearing animals were congested and enlarged; they weighed from 0.19 to 1.46 gms. as compared to 0.11 to 0.60 gms. for the 30 animals without tumors. The average weight of the spleens of tumor bearing animals was 0.52 gms. and of the animals without tumors was 0.20 gms. (Chart III).

Microscopic examination of the spleens from anim .ls with tumors showed evidence of increased formation of erythrocytes and an increased number of macrophages. Microscopic examination of the adrenal glands from animals with tumors revealed vascular engorgement of the sinusoids of the cortico-medullary area and destruction of the adjacent cortical cells (Fig. 3).

The uteri of animals with tumors were enlarged and did not show the usual atrophy of castration; their weights ranged from 0.08 to 0.23 gms., averaging 0.14 gms. Microscopic examination of these uteri revealed metaplasia of the epithelium and definite estrogenic effects were observed in smears of vaginal secretions. The uteri of animals without tumors showed the usual atrophy of castration.

The seminal vesicles and prostate of male animals with tumors also showed evidence of stimulation; their weights varied from 0.02 to 0.17 gms., averaging 0.10 gms. Microscopic examination showed secretion in these seminal vesicles.

Another unusual change was the presence of hemorrhagic lymph nodules in the submaxillary glands in the male animals bearing large tumors. These nodules showed markedly dilated blood channels and evidence of hemopoiesis.

DISCUSSION

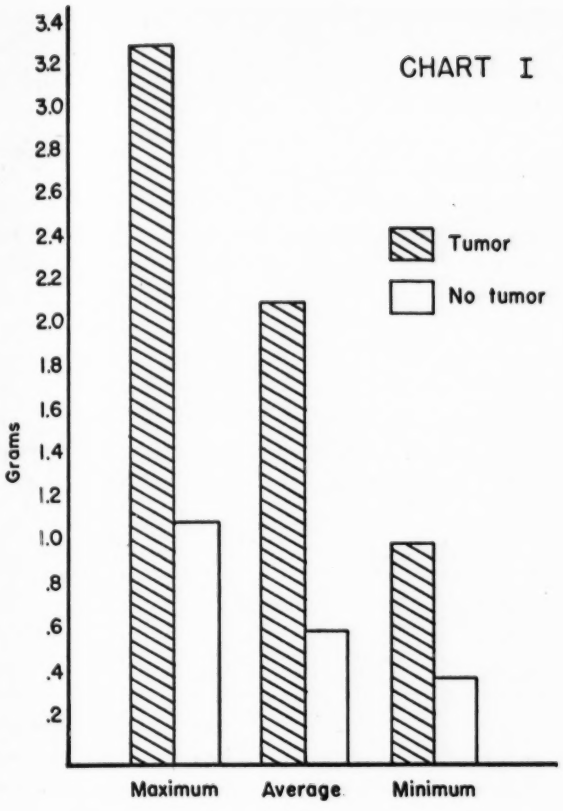
An analysis of the results presented indicates that the most significant difference between the tumor bearing and the non tumor bearing animals is in the blood weight. The difference in the weight of the spleen is next in significance, followed by the weight of the liver. This would indicate that the primary change of the condition described is in the blood volume. Further studies are necessary to discover whether this is due to a fluid volume change resulting from water retention. The observations previously reported indicate that only slight hemodilution occurs (5).

The changes described are not dissimilar to those found in pregnancy. The increase in weight of pregnant mice is greater than can be accounted for by the products of conception (1). Weight loss in excess of the weights of the uterine contents occurs in the first 24 hours following delivery and is probably due primarily to loss of body water. Weights of pregnant mice after killing the fetuses by pressure are maintained so long as both placentae and ovaries are present but the absence of either one causes a definite weight loss (7, 8). These changes are not unlike the loss of weight in animals from which the tumors were removed.

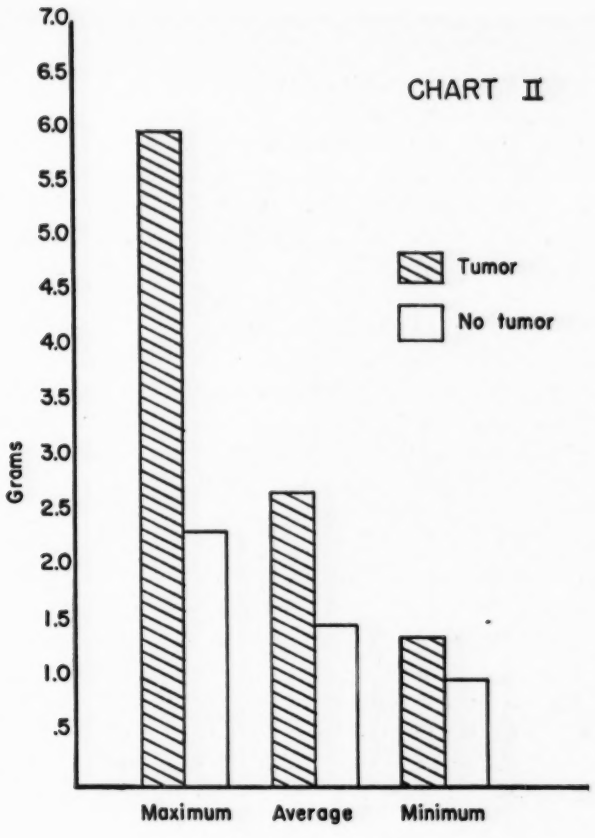
In women there is a progressive increase in the plasma volume from early pregnancy through the ninth lunar month, amounting to an increase of 65 per cent above the average non-pregnant value and 2 to 3 weeks before delivery it decreases to approximately 50 per cent above the non-pregnant value. It has been stated that "some hormonal influence may affect the blood volume changes observed in pregnant women" (9).

Although the transplanted ovarian tumor undoubtedly produces a substance with an estrogenic effect, as indicated by the changes in the uteri and the vaginal smears, it seems unlikely that the condition of hypervolemia could be attributed to estrogen. There is no evidence that estrogen alone or in combination with progesterone significantly alters blood volume.

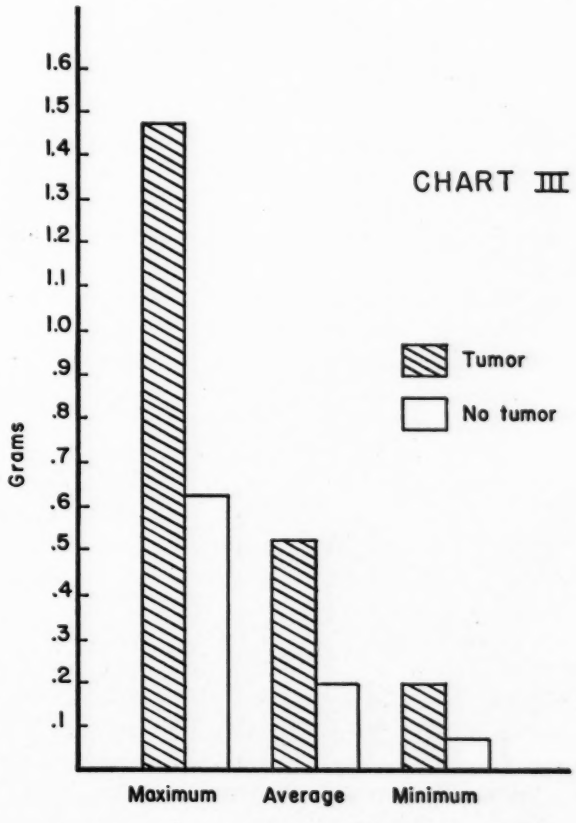
The hormonal effect noted is not entirely due to estrogen as enlargement of the seminal vesicles also occurs. The changes are not due to the crude compound as tumor bearing animals which did not



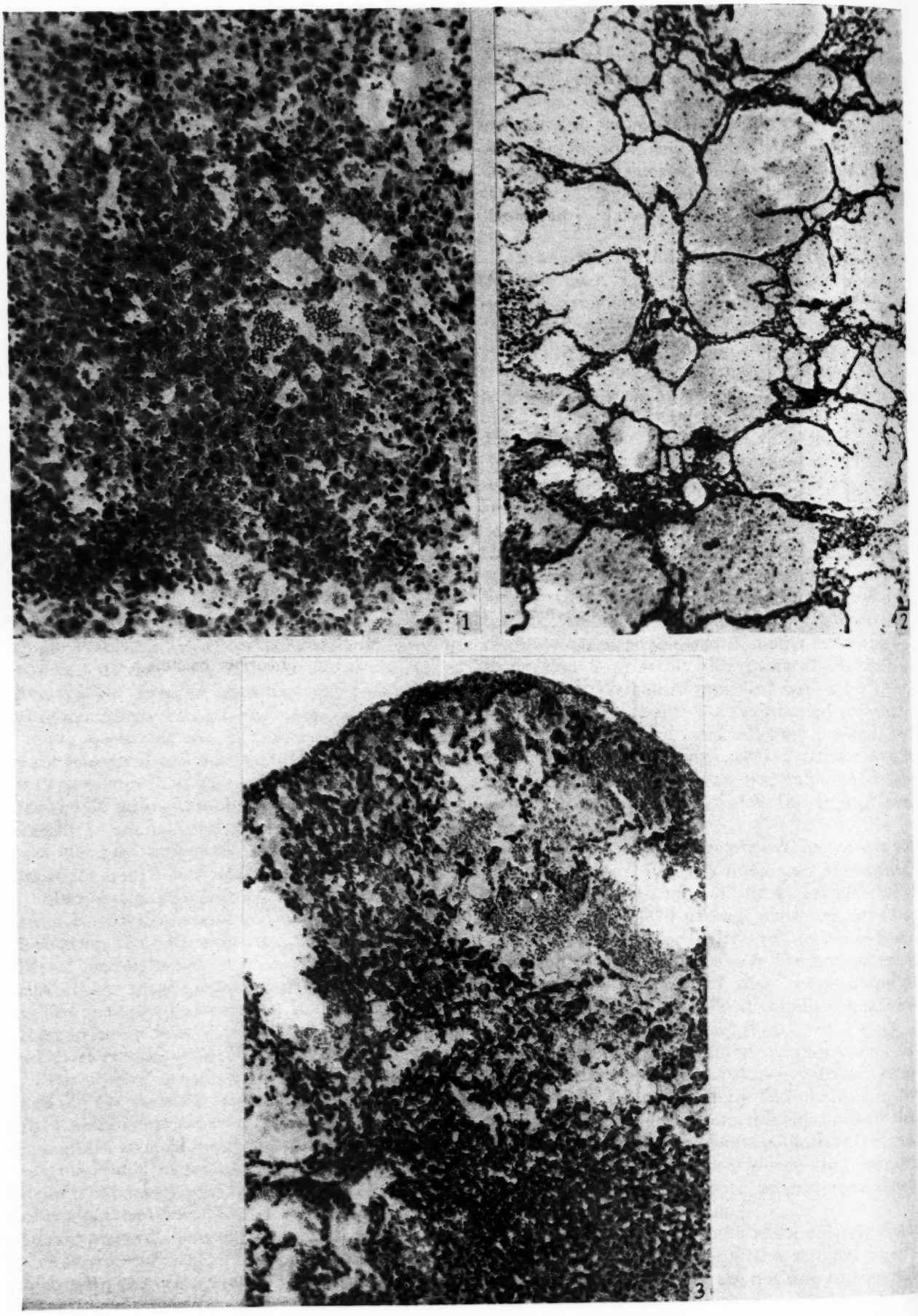
Blood weight in tumor and non-tumor bearing animals



Weight of liver in tumor and non-tumor bearing animals



Weight of spleen in tumor and non-tumor bearing animals



FIGS. 1–3

receive any hormonal treatment developed the same changes and animals without tumors receiving the crude compound did not.

It is unlikely that these changes are due to infection as reversion in weight occurs in those animals operated upon with removal of the tumor and also by the fact that these changes do not occur in a twin of a parabion having a transplanted tumor (unpublished data).

SUMMARY

1. Mice bearing a transplanted granulosa cell tumor have hypervolemia and associated sinusoidal dilatation in the liver, spleen, and adrenal glands.

2. An estrogenic effect, as evidenced by vaginal smears and enlargement of the uteri, was noted in castrated female animals bearing this tumor.

3. Enlargement of the seminal vesicles and prostate was observed in castrated, tumor bearing males.

REFERENCES

1. BROOKSBY, J. B., and NEWTON, W. H. Effect of the Placenta on the Body Weight of the Mouse. J. Physiol., **92**:136–150, 1938.
2. CLIFFTON, E., and PAN, S. The Effect of a Progesterone Compound on the Growth of a Transplanted Granulosa Cell Tumor. Proc. Soc. Exper. Biol. and Med., **69**:516–518, 1948.
3. FURTH, J. Transplantation of Induced Granulosa Cell Tumors and Luteoma in Mice. Secondary Effects of These Growths. Proc. Soc. Exper. Biol. and Med., **61**:212–214, 1946.
4. FURTH, J., and BOON, M. C. Liver Changes Associated with a Transplanted Granulosa Cell Carcinoma in Mice. Proc. Soc. Exper. Biol. and Med., **58**:112–114, 1945.
5. FURTH, J., and SOBEL, H. Hypervolemia Secondary to Grafted Granulosa Cell Tumors. J. Nat. Cancer Inst., **7**: 103–113, 1946.
6. FURTH, J., and SOBEL, H. Neoplastic Transformation of Granulosa Cells in Grafts of Normal Ovaries into Spleens of Gonadectomized Mice. J. Nat. Cancer Inst., **8**:7–16, 1947.
7. NEWTON, W. H., and LITS, F. J. Pseudo-parturition in the Mouse and the Relation of the Placenta to Post-partum Oestrus. J. Physiol., **84**:196–207, 1935.
8. NEWTON, W. H., and LITS, F. J. Criteria of Placental Endocrine Activity in the Mouse. Anat. Rec., **72**:333–349, 1938.
9. THOMPSON, K. J., HIRSHEIMER, A., GIBSON, J. G., and EVANS, W. A. Studies on the Circulation in Pregnancy. Am. J. Obst. and Gynec., **36**:48–59, 1938.

FIG. 1.—Section of liver of tumor bearing animal No. 106 C57 showing early sinusoidal dilatation. Mag. 16 mm. objective and 10× ocular.

FIG. 2.—Section of liver of tumor bearing animal B-1C57 showing marked sinusoidal dilatation. Mag. 16 mm. objective and 10× ocular.

FIG. 3.—Section of adrenal gland of tumor bearing animal B-1 C57 showing sinusoidal dilatation. Mag. 16 mm. objective and 10× ocular.

Studies on the Protein-bound Aminoazo Dyes Formed *in vivo* from 4-Dimethylaminoazobenzene and Its C-Monomethyl Derivatives*

E. C. Miller, Ph.D., J. A. Miller, Ph.D.,
R. W. Sapp, B.S., and G. M. Weber, B.S.

(*From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wisconsin*)

It appears to be a reasonable assumption that some of the steps of the carcinogenic process induced by a given carcinogen are at least similar to those involved in the carcinogenic processes induced by other types of carcinogens (including the agents responsible for "spontaneous" tumors). For this reason we have chosen to study intensively one carcinogenic process in particular, *i.e.* the induction of liver tumors in rats by 4-dimethylaminoazobenzene and its derivatives. This carcinogenic process appears to be particularly amenable to biochemical analysis (3, 4, 6 to 11, 13 to 19). Recently, protein-bound aminoazo derivatives of 4-dimethylaminoazobenzene were found in the livers of rats fed the carcinogen for periods of a few days to several months (3). The amount of protein-bound dye was found to vary with the period for which the carcinogen had been fed, *i.e.*, a maximum level was reached after 3 to 6 weeks and thereafter the level decreased until at the time of appearance of gross tumors only about half of the maximum level was present in the non-tumorous portions of the livers. However, no protein-bound dye could be detected in the tumors; this difference can be interpreted as representing a protein difference between liver and liver tumor. These observations and certain correlations noted between the presence of or level of protein-bound dye and the susceptibility of various species or of various tissues in the rat to the induction of tumors (3) suggest strongly that the combination between aminoazo dye and liver protein constitutes one of the first steps in this carcinogenic process. The tumor cells could be regarded as the final result of the continual depletion or alteration of proteins by the dye in certain of the surviving liver cells.

Each of the various C-monomethyl derivatives of 4-dimethylaminoazobenzene, which have widely different carcinogenic activities (Fig. 1 and [7]), also gives rise to considerable levels of protein-bound dye in the liver of the rat (9). Estimations of the molar level of each bound dye are not necessary to make comparisons between the carcinogenicity of the dyes and the times required to reach the maximal level of bound dye. However, no accurate comparisons could be made between carcinogenic activity and the level of bound dye formed until methods could be devised to estimate the molar levels of the bound dyes in these livers. This paper deals with these aspects of the protein-bound dyes.

METHODS

Care of the animals.—Male rats of the Sprague-Dawley strain and weighing 160 to 210 gm. were housed in screen-bottom cages in groups of 4 to 8. Food and water were available *ad libitum*. The basal diet (16) used in all of these experiments contains 12 per cent crude casein, 2 per cent Vitab rice bran extract, 4 per cent salts, 77 per cent glucose monohydrate (cerelose), and 5 per cent corn oil. The azo dyes (4, 7) were added to the diets after solution with heat in the corn oil. By fluorometric analysis (2) the diets contained 0.5 to 0.8 mgm. of riboflavin per kgm. Each rat received 1 drop of halibut liver oil per month.

Bound dye analyses.—The rats were killed with ether and their livers perfused *in situ* as previously described (3). The excised livers were homogenized in 4 volumes of distilled water in a Waring blendor and the homogenate diluted to contain 15 gm. of liver per 100 ml. A convenient quantity (usually equivalent to about 2 gm. of liver) was precipitated with an equal volume of 20 per cent trichloroacetic acid, washed successively with 1 *M* pH 5 acetate buffer and 95 per cent ethanol, and extracted with 95 per cent ethanol at 60° C. in a Soxhlet apparatus for 48 hours (3). The extracted samples of protein-nucleoprotein were dried *in vacuo* over $CaCl_2$ until most of the ethanol had been removed and then finally dried over sulfuric acid. Analyses for non-polar, polar, and total protein-bound dyes were carried out as before (3) except that the extracts were

* This investigation was aided by grants from the National Cancer Institute, the Jane Coffin Childs Fund for Medical Research, and the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

evaporated to dryness *in vacuo* in 125 ml. Erlenmeyer flasks. It is essential that the temperature of the oil bath used for the hydrolysis be maintained at 80 ± 1° C.

The levels of bound dye were observed qualitatively soon after the sacrifice of the animals by fixing 1 mm. thick cross sections of the perfused livers in a mixture of formalin, acetic acid, ethanol, and water in the proportions of 1:1:3:5 respectively. After 5 minutes the hardened and bleached sections were blotted and transferred to 10 per cent trichloroacetic acid. Five minutes after the sections ceased to float they generally had assumed a maximum pink coloration due to the aminoazo dyes present. Since the majority of the dyes present are protein-bound (3) and since the qualitative observation of the colored slices agreed roughly with the quantitative determinations made later on the same livers the above test serves as a rapid method of ascertaining the approximate level of protein-bound dye in the liver.

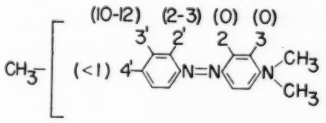


FIG. 1.—The observed carcinogenic activities (7) of various C-monomethyl derivatives of 4-dimethylaminoazobenzene (assigned activity of parent compound = 6). The activities are given in parentheses next to the position numbers on the rings.

Bound dye concentrates.—Since large scale liberation of the bound dyes by alkaline hydrolysis has so far yielded lower quantities of dye and higher blanks than are obtained with 50 mgm. samples (3), the dye samples remaining from the analytical studies have generally been pooled and stored at 0° C. When insufficient dye for characterization was available from this source, numerous 50 mgm. lots of protein were hydrolyzed and pooled prior to extraction. Concentrates of the polar bound dye were prepared by making the acid-ethanol solutions strongly alkaline with potassium hydroxide and diluting to an ethanol concentration of 25 per cent. The non-polar dyes were removed by 3 extractions with an equal volume of light petroleum ether (Skelly Solve B, b.p. 66 to 68° C.) and the completeness of extraction was tested by shaking the last extract with a few ml. of 7 *N* HCl. The aqueous solution was extracted first with 0.7 volumes of 1:7 ethanol-ethyl ether and then with 0.3 volumes of 1:5 ethanol-ethyl ether. The combined ethereal extracts were evaporated to near-dryness *in vacuo*, diluted to a known volume with ethanol, and stored at 0° C. Similar preparations from the liver protein of rats fed the basal diet served as controls and were termed basal extracts.

Determination of monophenyl amines derived from aminoazo dyes.—In the course of a search for means of determining quantitatively the monophenyl amines derived by reduction of the various aminoazo dyes, it was decided to make use of the old observation of Böniger (1) that aromatic amines react readily with sodium β-naphthoquinone-4-sulfonate at room temperature and at neutrality to yield highly colored Schiff bases. The equation for the reaction of a typical aromatic amine, aniline, with this reagent is shown in Figure 2. It was found that the Schiff bases of dimethyl-*p*-phenylene diamine, monomethyl-*p*-phenylene diamine, *p*-phenylene diamine, *p*-aminophenol, *o*-aminophenol, and aniline could be differentiated by their solubility characteristics and light absorption maxima. These are the amines obtained by the reduction of 4-dimethylaminoazobenzene, and its known or possible metabolites (4 to 8, 10) 4-monomethylaminoazobenzene, 4-aminoazobenzene, the 4′-hydroxy derivatives of these dyes, and 2′-hydroxy-4-dimethylaminoazobenzene. By application of the procedures detailed below it was further shown that each of the aminoazo dyes listed above was quantitatively reduced to its two constituent amines by refluxing about 20 μgm. of the dye in 10 ml. of 1 *N* HCl in the presence of about 0.05 gm. of mossy tin for 30 minutes.

For analysis of the mixed amines the reduction mixture from 20 μgm. of dye was cooled, 5 ml. of 0.2 *M* phosphate buffer, pH 7, were added, and the pH of the solution was quickly adjusted to pH 7 ± 0.2 with 11 *N* KOH.[1] Five ml. of a 0.1 per cent solution of sodium β-naphthoquinone-4-sulfonate in the phosphate buffer (prepared just prior to use) and 5 ml. of benzene were added in that order and the mixture shaken vigorously in a glass-stoppered flask for 1 minute. Since the Schiff bases possess both basic and acidic properties (Fig. 2) and furthermore are easily hydrolyzed, care should be taken to keep the pH of this reaction close to 7 if quantitative extraction and maximum stability are to be achieved. The Schiff bases of dimethyl-*p*-phenylene diamine, monomethyl-*p*-phenylene diamine, *o*-aminophenol, and aniline were quantitatively extracted into the benzene phase. The Schiff bases of the dimethyl- and monomethyl-*p*-phenylene diamines absorbed maximally at 580 and 570 mμ, respectively, in the benzene solution. It was found that the Schiff base of monomethyl-*p*-phenylene diamine was selectively destroyed by acetic anhydride. Hence the sum of the two bases was read at 575 mμ, then 0.1 ml. of acetic anhydride was added to 3 ml. of the benzene solution and the residual absorption due to dimethyl-*p*-phenylene diamine was determined at the same wave length. The amounts of *o*-aminophenol and aniline were calculated by determining the light absorption at 480 mμ (the maximum for the *o*-aminophenol derivative) and 450 mμ (the maximum for the aniline derivative) prior to the addition of acetic anhydride, subtracting the small absorption due to the methylated diamines at these wave lengths, and finally applying simultaneous equations.

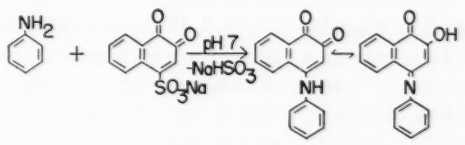


FIG. 2.—The Böniger Reaction

p-Phenylene diamine formed a Schiff base which even at microgram levels was highly insoluble in every organic solvent tried; however, it was soluble in dilute aqueous

[1] Hydrion pH paper, Micro Essential Laboratory, Brooklyn, N.Y.

alkali. Generally it collected as a red film at the interface in the extraction of the Schiff bases of the above amines by benzene. After the clear benzene layer was removed the remaining aqueous suspension was transferred to a Gooch crucible and quantitatively collected on an asbestos pad[2] and washed with three 2 ml. portions of the phosphate buffer. For estimation the asbestos pad, together with small wads of asbestos used in wiping the residual precipitate from the walls of the crucible were transferred to a centrifuge tube containing 10 ml. of 0.1 *N* NaOH. After elution of the base and strong centrifugation the absorption of the alkaline solution was determined at 490 mμ. The Schiff base of *p*-aminophenol remained in the benzene-extracted and filtered solution. This base was extracted into 6 ml. of *n*-amyl acetate;[3] this solution absorbed maximally at 480 mμ.

When *m*-aminophenol, obtained for example by the reduction of 3′-hydroxy dyes, was present about half of the Schiff base of this amine was extracted into the benzene and the remainder appeared in the *n*-amyl acetate extract. It could easily be distinguished from aniline since it is not steam volatile from alkali. Approximate corrections for its presence in the *n*-amyl acetate extract could be made since its Schiff base absorbed maximally at 450 mμ.

When 2′-, 3′-, or 4′-methyl-4-dimethylaminoazobenzene was reduced and the resulting amines analyzed, the Schiff base of *o*-, *m*-, or *p*-toluidine was found in the benzene solution; these bases absorbed maximally at 460 mμ.

While tissue constituents or derivatives such as ammonia and amino acids also react with the quinone, the products formed are too polar to be easily extracted by benzene or amyl acetate. Thus in this reaction the ether-ethanol extracts of the alkaline hydrolysates of normal liver protein (*i.e.* the basal extracts) yielded only low levels of yellow compounds extractable into benzene and amyl acetate.

In some cases a more rigorous characterization of the aniline or toluidine fraction was desirable. This was achieved by steam distillation of the mixture of amines from an all-glass apparatus after the addition of excess alkali and a little octadecyl alcohol as an anti-foam agent. The distillate was collected in a glass-stoppered flask containing the quinone in phosphate buffer and the volatile amine was determined colorimetrically after extraction into benzene. Aniline and the toluidines each distilled quantitatively under these conditions while none of the aminophenols considered above distilled at all. While *p*-phenylene diamine did not steam distill, the methylated diamines distilled with steam to an appreciable extent; however, as noted above the Schiff bases of the latter amines are easily distinguished from the bases derived from the monoamines.

A Ceuco-Sheard spectrophotelometer modified for the use of 13 × 100 mm. tubes containing 3 ml. of solution was used for all colorimetric and spectra determinations. The nominal slit width used was 10 mμ. Solutions of each Schiff base obeyed Beer's law, and the amount of base formed was proportional to the quantity of amine added in the range of 5 to 30 μgm. of the amines studied. When mixtures of amines or the reduction products of aminoazo dyes were analyzed, the recovery of the individual amines ranged from 90 to 95 per cent.

RESULTS

Identification of aniline in the reduction mixture of the polar bound dye from rats fed 4-dimethylaminoazobenzene.—While in this case the non-polar fraction of the protein-bound dye (about 10 per cent of the total) has been shown to be a mixture of 4-monomethylaminoazobenzene and 4-aminoazobenzene (3), the major fraction, the polar dye, remains to be identified. As an initial step towards its characterization this fraction was reduced and reacted with the quinone. The benzene extract of the reaction medium was yellow and had an absorption spectrum (after correction for the nonspecific absorption derived from the basal extract) similar to that of aniline with a maximum at 450 mμ. Similar results were obtained when the reduced dye was subjected to steam distillation prior to forming the Schiff base. No *p*-phenylene diamine or its methylated derivatives or any of the aminophenols could be detected by the methods outlined above. The amount of steam-volatile amine liberated was proportional to the amount of polar dye reduced. However, it was apparent that another and more polar amine had also reacted with the reagent, since the benzene-extracted reaction mixture of the reduced dye had a more intense color than the same reaction mixture from a reduced basal extract. A part of this color could be extracted with *n*-butanol, but this solvent also slowly extracted the excess quinone so that the absorption spectra of these extracts were unsatisfactory. The formation of two amines is, of course, required in the reduction of an azo linkage.

Ratio of non-polar to polar bound dye in the liver protein of rats fed the C-monomethyl derivatives of 4-dimethylaminoazobenzene.—Fractionation (3) of the total bound dye from the livers of rats fed each of the C-monomethyl dyes showed that in each case about 5 to 10 per cent was non-polar and the remaining 90 to 95 per cent was polar in character. This distribution is similar to that found for the parent dye (3).

Reductive cleavage of the polar bound dyes derived from the C-monomethyl derivatives of 4-dimethyla-

[2] Acid-washed long staple asbestos was used. It was cut with scissors into approximately ½ cm. lengths, suspended in distilled water, and centrifuged lightly several times to remove the small difficultly sedimentable fibers.

[3] The amyl acetate must be free of amyl alcohol if low tissue blanks are to be obtained. If it is recovered after shaking with aqueous media it must be carefully fractionated and only the fraction boiling at 145 to 147° C. used. The yields of recovered ester are low, however, since the ester and alcohol form a constant boiling mixture which distills at about 137° C.

minoazobenzene.—Reductive cleavage of each of the polar bound dyes derived from the 2-, 2′-, 3′-, or 4′-methyl derivatives of 4-dimethylaminoazobenzene or 3-methyl-4-monomethylaminoazobenzene also gave rise to a steam-distillable amine which formed a benzene-soluble Schiff base. Thus, it appears that aniline or the appropriate toluidine was liberated from each bound dye. This was substantiated by a relatively good agreement between the absorption curves of these Schiff bases after correction for the absorption of the basal extract and the curves of the bases from the authentic amines. As in the case of the bound dye from 4-dimethylaminoazobenzene a more polar amine was also formed by the reduction of each bound dye.

Calculation of the micromolar levels of the bound dyes.—On the assumptions that each of the bound dyes was quantitatively reduced under the conditions used and that one mole of aniline or the corresponding toluidine was the steam-volatile amine formed by the reduction of each mole of these dyes, it is possible to calculate the micromolar levels of each bound dye in terms of the micromoles of steam-distillable amine obtained by reduction.[4] These assumptions appear reasonable at the present time. Accordingly, in order to compare the levels of bound dyes of rats fed various dyes, the bound dye levels per 100 mgm. protein have been expressed in micromoles on the following basis:

$$\frac{E_{\text{dye-fed}} - E_{\text{basal}}}{E_{\text{equivalent to 1 } \mu\text{M of amine}}} = \mu\text{M of bound dye}$$

where $E = \log \frac{I_0}{I}$ at 520 mμ for a solution of the dye or basal extracts in a mixture of 2 ml. of 95 per cent ethanol and 2.5 ml. of 7*N* HCl (3). Under our conditions the values for E which were equivalent to 1 μM of steam-distillable amine were 12.0, 12.7, 9.7, 16.0, 13.4, and 8.4 for the amines derived from 4-dimethylaminoazobenzene, 3-methyl-4-monomethylaminoazobenzene, 2'-methyl-, 3'-methyl-, 4'-methyl-, and 2-methyl-4-dimethylaminoazobenzene, respectively.

[4] As noted above the reduction mixtures from each of the polar bound dyes contained an unidentified polar amine fraction. Since the reduction of an azo linkage requires the formation of 2 amines, the polar amine fraction has, for the present, been assumed to be derived from the diamine portion of the dye. However, if the polar amine fraction from one or more bound dyes should also contain an amine derived from the non-diamine fraction, the equivalences obtained above would be subject to an undetermined error. Such a polar amine fraction might, for example, be formed by oxidation of the C-methyl group to a carboxyl group. The Schiff bases of *o*-, *m*-, and *p*-aminobenzoic acid were not appreciably extracted from aqueous solution into either benzene or amyl acetate. In these tests 100 μgm. of each amine were used.

Bound dye levels in the livers of rats fed the C-monomethyl derivatives of 4-dimethylaminoazobenzene.—Since earlier work showed that the level of bound dye increased for the first 3 to 6 weeks that 4-dimethylaminoazobenzene was fed and thereafter decreased, it was of interest to determine the bound dye-time curves for each of the C-monomethyl derivatives. These results are presented in Figures 3 and 4. The dye values are the total bound dye expressed in micromoles per 100 mgm. of protein and each point is the average of 3 rats analyzed individually. Typical ranges have been presented previously (3). The experiments were conducted in series, and each was controlled by a group of rats receiving 4-dimethylaminoazobenzene at an equimolar level to the other dyes fed. Such a control is necessary since the levels of dye obtained and the time at which the maximum is attained vary somewhat from one series to another.

When the dyes with the methyl group on the prime ring are considered, two correlations are evident. First, the more active the dye the more rapidly the maximum level of bound dye was reached. Thus, the times before the maximum level was attained were 2, 4, 8, and $\geq$21 for 3′-methyl-4-dimethylaminoazobenzene (activity = 10 to 12), 4-dimethylaminoazobenzene (activity = 6), 2′-methyl-4-dimethylaminoazobenzene (activity = 2 to 3), and 4′-methyl-4-dimethylaminoazobenzene (activity < 1), respectively (Fig. 3). Second, during the first 4 weeks the slopes of the bound dye-time curves were roughly in direct proportion to the carcinogenicities of the dyes fed. After the curves began to fall, no further correlation of dye level and carcinogenicity was possible. The data for 4-dimethylaminoazobenzene and its 3′-methyl and 4′-methyl derivatives were obtained in the same series. The 2′-methyl-4-dimethylaminoazobenzene data were taken from another series in which the curve for 4-dimethylaminoazobenzene reached a maximum at 3 weeks, but they are included in Figure 3 to facilitate comparisons. Similar results were obtained in a second series in which the maximum levels of bound dye occurred after 2, 3, and $\geq$16 weeks of dye-feeding with 3′-methyl-4-dimethylaminoazobenzene, 4-dimethylaminoazobenzene, and 4′-methyl-4-dimethylaminoazobenzene, respectively. In this experiment 2′-methyl-4-dimethylaminoazobenzene was fed for only 6 weeks, and the level of bound dye did not reach a maximum in this time. The micromolar levels of bound dye again increased with the carcinogenicity of the dye during the first 4 weeks of the experiment. The molar levels of the dyes fed in

these experiments were equivalent to 0.058 per cent of 4-dimethylaminoazobenzene.

When the dyes with a methyl substituent on the same ring with the $-N(CH_3)_2$ group were fed, only the time at which the maximum level of bound dye was reached and not its micromolar

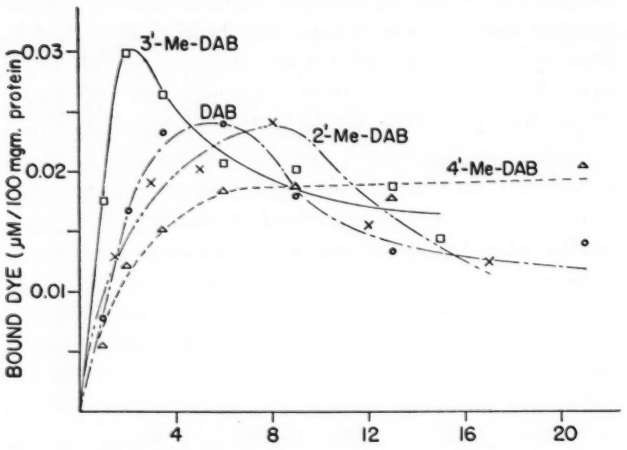


FIG. 3.—The levels of bound dye in the livers of rats fed 4-dimethylaminoazobenzene (DAB) or certain of its C-monomethyl derivatives.

level appeared to correlate with the carcinogenicity of the compounds (Fig. 4). These dyes were fed at a level equimolar to 0.06 per cent of 4-dimethylaminoazobenzene, and 3-methyl-4-monomethylaminoazobenzene was used instead of the dimethyl compound since 3-methyl-4-dimethylaminoazobenzene is difficult to prepare and the two compounds are similar in having extremely low carcinogenic activity (7). The equal carcinogenicity of N-monomethyl- and N-dimethylaminoazo dyes has also been demonstrated for 4-dimethylaminoazobenzene, its 2'-, 3'-, and 4'-methyl derivatives, and their monomethyl derivatives (4, 7, 18, 19). Whether the 2- or 3-methyl dye was fed, the level of bound dye reached a maximum after 12 weeks of dye-feeding and then fell off slightly while the bound dye level of the livers from rats fed 4-dimethylaminoazobenzene reached its peak in only 3 weeks. In other experiments where 2-methyl-4-dimethylaminoazobenzene was fed for 6 or 12 weeks, the level of bound dye rose throughout the dye-feeding period.

Bound dye levels in the livers of rats fed different amounts of 4-dimethylaminoazobenzene.—In addition to feeding azo dyes of different activities, graded carcinogenic responses can also be obtained by feeding several levels of a given carcinogen. When the levels of bound dye were determined in the livers of rats fed 0.060, 0.045, and 0.030 per cent of 4-dimethylaminoazobenzene, in one experiment the maxima, were reached after 2, 4, and 8 weeks, respectively (Fig. 5). The levels of bound dye during the first 4 week period increased as higher levels were included in the diets, but not in proportion to the amounts fed. In another series the maximum level of bound dye was found after 3 weeks for rats fed 0.060 or 0.045 per cent and after 8 weeks for those fed only 0.030 per cent. The tumor incidences obtained when 0.045 and 0.030 per cent levels of 4-dimethylaminoazobenzene are fed are usually 80 to 100 per cent and 0 to 15 per cent, respectively, of that obtained with 0.060 per cent (5).

DISCUSSION

Our earlier observations (3) on the *in vivo* chemical combination of derivatives of 4-dimethylaminoazobenzene and 4-monomethylaminoazobenzene with the liver protein of rats have now been extended to include the C-monomethyl dyes. In all of these cases 5 to 10 per cent of the bound dyes are non-polar in character, and the non-polar fraction derived from 4-dimethylaminoazobenzene has been characterized as a mixture of 4-monomethylaminoazobenzene and 4-aminoazobenzene. The structure of the polar bound dyes remains uncertain, but some deductions are possible. Thus, the azo linkage and the 4-amino group must both be present since the dyes show the reversible color change from red in strong acid to yellow in alkali or organic solvents which is characteristic of 4-aminoazo dyes but not of azo-

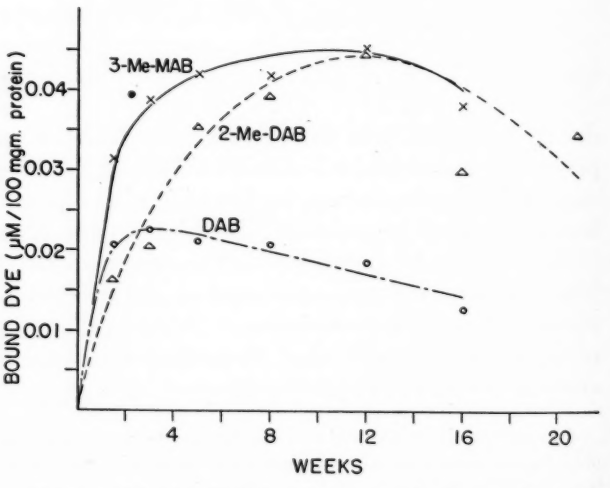


FIG. 4.—The levels of bound dye in the livers of rats fed 4-dimethylaminoazobenzene (DAB) or certain of its C-monomethyl derivatives.

benzene (9). Further, the absorption maximum at 523 mμ for the bound dye derived from 4-dimethylaminoazobenzene suggests the presence of 1 or 2 alkyl substituents on the amino group since the absorption maximum in acid solution shifts from 500 mμ for 4-aminoazobenzene to 518 mμ for the

dimethyl compound (3). The absorption maxima for the bound dyes derived from the C-monomethyl derivatives also correspond to those of alkylated aminoazo dyes (9). The liberation of aniline or a toluidine by the reduction of each of the polar bound dyes with tin and hot acid indicates that the prime ring is unaltered by the protein-binding reaction. Hence the dye must be combined with the protein either through a derivative of the $-N(CH_3)_2$ group or through some substituent on the ring bearing this group. The presence of an unidentified polar amine in the reduction mixture supports this conclusion.

Until the polar bound dye is characterized further, approximate means of estimating the amount of these dyes must be used. One possible method involves the assumption that the color potency of each bound dye is relatively close to that of the dye fed; in this case the level of bound dye might be calculated in terms of the constants for the free dyes. Such calculations are always open to doubt since relatively small structural changes can alter the light absorption characteristics of these dyes considerably (9). Probably a more reliable method is the one which depends on the quantities of aniline or toluidine formed on reduction of the bound dyes. While direct proof of a quantitative cleavage of the bound dyes is not available, the demonstrated quantitative cleavage of a number of different aminoazo dyes and the liberation of proportional quantities of aniline from different amounts of the bound dye derived from 4-dimethylaminoazobenzene suggest its reliability. When the levels of bound dye as calculated by each of these methods are compared, the values obtained by the absorption of the acid solutions at 520 mμ are 139, 169, 177, 478, 82, and 121 per cent of those obtained from the analyses for steam-distillable amines for 4-dimethylaminoazobenzene, its 4′-, 3′-, 2′-, and 2-methyl derivatives, and 3-methyl-4-monomethylaminoazobenzene, respectively. The large deviation in the case of the 2′-methyl compound is probably related to the anomalous absorption spectrum of the parent compound (9). The smaller discrepancies between the two types of calculations for the other bound dyes may also be related to alterations in the absorption characteristics on binding with protein.

The correlations obtained earlier (3) between the level of bound dye derived from 4-dimethylaminoazobenzene and the tumor incidences obtained with various species[5] of animals, different tissues of the rat, and the livers of rats fed diets high and low in riboflavin suggested that the binding of the dye to liver protein constitutes one of the first steps in the carcinogenic process. It appears likely that such a protein derivative would lack the physiological usefulness of the parent protein and, furthermore, the enzyme protein responsible for metabolizing the ingested dye to a protein-combining form might be among the first to be attacked by the reactive dye. Quantitative changes in the protein content of the livers subjected to the dye have been shown to occur. Analyses of the cellular particulates and soluble proteins of the liver after feeding either 4-dimethylaminoazobenzene or its 3′-methyl derivative for 1 month show that the protein content of the nuclear fraction increases while that of the large and small granules decreases (13, 15). Even larger changes in the same

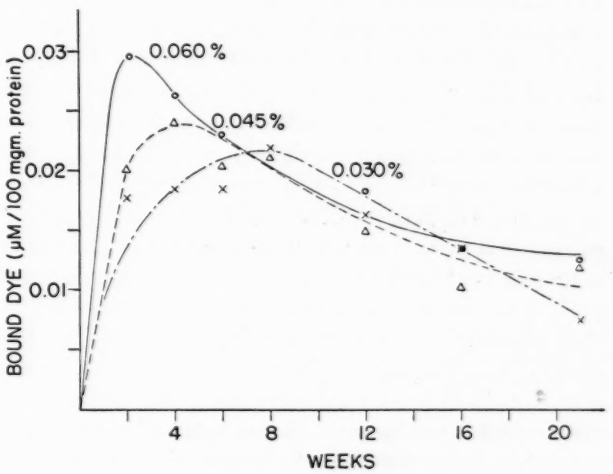


FIG. 5.—The levels of bound dye in the livers of rats fed various levels of 4-dimethylaminoazobenzene.

direction were found on analysis of the tumors (14). Of course, very important qualitative changes might be too subtle to be demonstrated in this gross manner, for when the bound dye derived from 4-dimethylaminoazobenzene is at a maximum only 3 per cent of the proteins could carry 1 molecule of dye per molecule if the average molecular weight of the liver proteins was assumed to be 100,000. If a higher molecular weight were assumed, a higher percentage of the total protein molecules would be required for a 1:1 molar ratio and conversely a lower percentage of dye-protein would be present if on the average more than one molecule of dye were bound to a molecule of protein. However, since there is a relatively rapid turnover of protein-bound dye in the liver

[5] In addition to the 4 species previously reported (3) which are highly resistant to the dye and which also do not form protein-bound dye, we have found that the golden hamster also does not form any protein-bound dye even after the dye is fed for many months. This agrees with the very high resistance of this species to the carcinogenic action of 4-dimethylaminoazobenzene (Dr. P. N. Harris, private communication; unpublished tests in this laboratory).

(half-life = approximately 3 to 4 days, see [3]) significant quantities of protein could be affected during the several months needed to induce tumors by feeding of these dyes.

It would appear from the curves given in Figures 3 to 5 that the time at which the maximum concentration of protein-bound dye is reached is relatively more important with regard to the eventual formation of tumors than is the molar level of bound dye that is eventually attained. For example, the very weak carcinogens 4′-methyl-4-dimethylaminoazobenzene, 2-methyl-4-dimethylaminoazobenzene, and 3-methyl-4-monomethylaminoazobenzene form levels of bound dye which eventually exceed that formed by the reference compound, 4-dimethylaminoazobenzene. However, the former dyes form maximum levels of bound dye much later than does the more active parent dye. The same holds true when lower and hence less carcinogenic levels of 4-dimethylaminoazobenzene are fed. This is explicable if one regards the ascending portion of the bound dye-time curve as a period in which a majority of the liver cells can synthesize the proteins which become attached to the dye faster than they are removed by dye-binding and still in sufficient amounts for the normal functioning of the tissue. When a plateau is maintained for a long time, as in the case of the very weak carcinogen 4′-methyl-4-dimethylaminoazobenzene, the protein synthesis mechanisms of the cell are presumably just keeping pace with the losses and hence the changes leading to neoplasia are forestalled. It is of interest that 4′-methyl-4-dimethylaminoazobenzene eventually did produce a high percentage of liver tumors in rats when the dye was fed at levels and for times that were 2 to 3 times greater than those employed here (11). However, when the rate of synthesis of the proteins bound to the dye cannot keep pace with the combined demands of normal function and removal by the carcinogen, the level of protein-bound dye will fall despite the continued administration of the carcinogen; the rate of decrease appears to be about the same regardless of the time at which the maximum level is attained. Such a situation might result if the multiplication of these proteins was slower than the multiplication of the liver cells in a manner analogous to that found by Preer (12) for the kappa particle in *Paramecium aurelia* under certain conditions. Many cells may die as a result. However, among those that can survive some may lose the ability to make the proteins bound by the carcinogen and it is possible that this loss and the ability to survive are synonymous. If these proteins had been concerned with either intrinsic growth controls (*i.e.*, through competitive systems) or extrinsic growth controls (*i.e.*, hormonal influences) or both, the surviving cell could not respond to these normal controls. Such cells, if their mechanisms for the utilization and synthesis of substances essential for continued life on any plane were relatively intact, could then respond to further nutrition only through continued growth. Thus, they would have become tumor cells. The inability of the liver tumor cell to form protein-bound dye (3) is in agreement with these speculations.

SUMMARY

1. Methods were developed for the quantitative estimation of 5 to 30 μgm. of aniline, *o*-aminophenol, *p*-aminophenol, *p*-phenylene diamine, monomethyl-*p*-phenylene diamine, and dimethyl-*p*-phenylene diamine either alone, in mixtures, or as the reduction products obtained from aminoazo dyes. In the absence of aniline either *o*-, *m*-, or *p*-toluidine could also be estimated. These determinations depended on the formation of Schiff bases between the amines and sodium β-naphthoquinone-4-sulfonate and the separate estimation of the colored derivatives by their different solubility properties and absorption spectra.

2. On reduction the polar bound dye isolated from the livers of rats fed 4-dimethylaminoazobenzene yielded an amine with the characteristics of aniline and an unidentified polar amine. Similarly, reduction of the 2′-, 3′-, and 4′-methyl derivatives of 4-dimethylaminoazobenzene yielded amines similar to *o*-, *m*-, and *p*-toluidine, respectively, and 2-methyl-4-dimethylaminoazobenzene and 3-methyl-4-monomethylaminoazobenzene appeared to give rise to aniline. These results suggest that the dyes are bound to the protein either through the $-N(CH_3)_2$ group or to the ring bearing this group.

3. The levels of bound dye in the livers of rats fed 4-dimethylaminoazobenzene, its 2-, 2′-, 3′-, and 4′-methyl derivatives, and 3-methyl-4-monomethylaminoazobenzene were determined after dye-feeding periods of 1 to 21 weeks. When the derivatives with the methyl group on the prime ring were considered, there was a striking inverse correlation between carcinogenic activity and the time required to reach a maximum level of bound dye. With relative carcinogenicities of 10 to 12, 6, 2 to 3, and <1 the maxima were attained in approximately 2, 4, 8, and ≥ 21 weeks for 3′-methyl-4-dimethylaminoazobenzene, 4-dimethylaminoazobenzene, 2′-methyl-4-dimethylaminoazobenzene, and 4′-methyl-4-dimethylaminoazobenzene, respectively. When these data were calculated to micromolar levels by estimating the

distillable amine, the amount of bound dye found until the maximum was reached increased with the carcinogenicity of the dye when the methyl group was located on the prime ring. The bound dye levels of livers from rats fed 2-methyl-4-dimethylaminoazobenzene (activity = 0) and 3-methyl-4-monomethylaminoazobenzene (activity < 1) reached maxima after about 12 weeks, but these dyes formed much higher micromolar levels of bound dye than did 4-dimethylaminoazobenzene.

4. The bound dye-time curves for rats fed 0.060, 0.045, and 0.030 per cent of 4-dimethylaminoazobenzene reached maxima after 2, 4, and 8 and 3, 3, and 8 weeks, respectively, in two series. Since 0.030 per cent of this dye induces tumors much more slowly than the higher levels, these data conform to the inverse relationship between carcinogenic activity and the time required to reach a maximum level of bound dye as found for the C-monomethyl derivatives.

5. It is suggested that the ascending portion of the bound dye-time curve represents a period in which the liver cells, on the average, can synthesize the proteins which are bound to the dye faster than they are removed by dye-binding and in amounts sufficient for the normal functioning of the tissue. However, once the level of bound dye begins to fall with continued dye-feeding, the dye-binding may have affected one or more specific synthetic mechanisms to such an extent that they cannot keep pace with the combined demands of normal function and removal of protein by the carcinogen. Viable cells may finally result which have completely lost those systems controlling normal growth and hence represent the initial tumor cells. Such a concept is in agreement with the observed absence of protein-bound dye in liver tumors formed during the continuous feeding of the azo dye.

REFERENCES

1. BÖNIGER, M. Ueber 1, 2-Amidonapthol-4-monosulfosäure und Derivate derselben. Ber. d. deutsch. chem. Ges., **27**: 23–30, 1894.
2. CONNER, R. T., and STRAUB, G. J. Combined Determination of Riboflavin and Thiamine in Food Products. Indust. and Engin. Chem., Anal. Ed., **13**:385–388, 1941.
3. MILLER, E. C., and MILLER, J. A. The Presence and Significance of Bound Aminoazo Dyes in the Livers of Rats Fed *p*-Dimethylaminoazobenzene. Cancer Research, **7**: 468–480, 1947.
4. MILLER, J. A., and BAUMANN, C. A. The Carcinogenicity of Certain Azo Dyes Related to *p*-Dimethylaminoazobenzene. Cancer Research, **5**:227–234, 1945.
5. MILLER, J. A., and MILLER, E. C. Unpublished results.
6. MILLER, J. A., and MILLER, E. C. The Metabolism and Carcinogenicity of *p*-Dimethylaminoazobenzene and Related Compounds in the Rat. Cancer Research, **6**:39–41, 1946.
7. MILLER, J. A., and MILLER, E. C. The Carcinogenicity of Certain Derivatives of *p*-Dimethylaminoazobenzene in the Rat. J. Exper. Med., **87**:139–156, 1948.
8. MILLER, J. A., MILLER, E. C., and BAUMANN, C. A. On the Methylation and Demethylation of Certain Carcinogenic Azo Dyes in the Rat. Cancer Research, **5**:162–168, 1945.
9. MILLER, J. A., SAPP, R. W., and MILLER, E. C. The Absorption Spectra of Certain Carcinogenic Aminoazo Dyes and the Protein-bound Derivatives Formed from These Dyes *in Vivo*. J. Am. Chem. Soc., **70**:3458–3463, 1948.
10. MUELLER, G. C., and MILLER, J. A. The Metabolism of 4-Dimethylaminoazobenzene by Rat Liver Homogenates. J. Biol. Chem., **176**:535–544, 1948.
11. NAGAO, N. Über die Fütterungsversuche mit 4′-Methyl-4-N-dimethylaminoazobenzol und weiteren Polymethylderivaten bei weissen Ratten. Gann, **35**:8–20, 1941.
12. PREER, J. R. Some Properties of a Genetic Cytoplasmic Factor in Paramecium. Proc. Nat. Acad. Sc., U.S., **32**:247–253, 1946.
13. PRICE, J. M., MILLER, E. C., and MILLER, J. A. The Intracellular Distribution of Protein, Nucleic Acids, Riboflavin, and Protein-bound Aminoazo Dye in the Livers of Rats Fed *p*-Dimethylaminoazobenzene. J. Biol. Chem., **173**: 345–353, 1948.
14. PRICE, J. M., MILLER, J. A., MILLER, E. C., and WEBER, G. M. Studies on the Intracellular Composition of Liver and Liver Tumor from Rats Fed 4-Dimethylaminoazobenzene. Cancer Research, **9**:96–102, 1949.
15. PRICE, J. M., MILLER, J. A., MILLER, E. C., and WEBER, G. M. Studies on the Intracellular Composition of the Liver in Rats Fed Various C-Monomethyl Derivatives of 4-Dimethylaminoazobenzene. In preparation.
16. RUSCH, H. P., BAUMANN, C. A., MILLER, J. A., and KLINE, B. E. Experimental Liver Tumors, In: F. R. Moulton, Am. A. Adv. Sc. Res. Conf. Cancer, pp. 267–287. Washington, D.C., 1945.
17. STEVENSON, E. S., DOBRINER, K., and RHOADS, C. P. The Metabolism of Dimethylaminoazobenzene (Butter Yellow) in Rats. Cancer Research, **2**:160–167, 1942.
18. SUGIURA, K. The Carcinogenicity of Certain Compounds Related to *p*-Dimethylaminoazobenzene. Cancer Research, **8**:141–144, 1948.
19. SUGIURA, K., HALTER, C. R., KENSLER, C. J., and RHOADS, C. P. Observations on Rats Fed with Compounds Related to Dimethylaminoazobenzene. Cancer Research, **5**:235–238, 1945.

Influence of Calcium and Magnesium on Eugenol-Induced Desquamation of Mucus Epithelium in Gastric Pouches*†

SHIRLEY D. KRAUS, PH.D., AND FRANKLIN HOLLANDER, PH.D.

(From the Gastroenterology Research Laboratory, The Mount Sinai Hospital, New York 29, New York)

In order to find a satisfactory topical stimulus for the study of the physiology of gastric mucus secretion, numerous agents have been investigated in this laboratory (9, 10, 11). Gentle massage, aqueous ether (saturated), 5 per cent aqueous clove oil emulsion, 50 per cent ethyl alcohol, distilled water, isotonic and hypertonic NaCl, and ½ to 5 per cent aqueous eugenol emulsions were all found to induce desquamation of the surface epithelium in addition to acting as mucigogues. The most effective of these mucus stimuli was 5 per cent eugenol, and this also produced huge amounts of desquamation. Since there is reason to believe that pure gastric mucus is cell-free (9), this associated phenomenon of decreased cellular cohesion is of interest in relation to the general problem of gastric mucus function, but particularly with those aspects of the problem concerned with the gastric mucous barrier as a protective mechanism against chemical and physical irritants. The latter considerations arise especially in any investigation which is concerned with an exogenous, topical agent as an etiological factor in adenocarcinoma of the stomach. Furthermore, the loosening of the cement substance which results in this diminished cohesion may well be related to the difference between normal and cancerous tissues, which makes for the invasive character of the latter.

Some of the attempts to discover a common denominator for such decreases in cellular cohesion have resolved themselves around the ionic constituents of the intercellular cement. Overton (14) stressed the importance of an easily dissociating calcium salt as the basis of the cohesive material which binds cellular membranes. A partial explanation for the decreased cohesiveness of malignant cells has been related to a calcium deficiency of the tissues by Brunschwig *et al.* (1) and by Coman (5). Further support for the concept that calcium plays a role in the maintenance of cellular cohesion has been fostered by the observations that calcium-free solutions decrease the cohesiveness of normal epithelial cells (6), capillary endothelium (3), ciliated gill cells of *Mytilus* (13), and blastomeres of developing sea urchins (8). Methylcholanthrene, which is known to decrease the cohesiveness of squamous epithelial cells, likewise decreases the calcium content of the mouse epidermis (2).

Since both calcium and magnesium are constituents of cell membranes (16), both ions may be involved in the maintenance of cellular cohesion in general. Some foundation for this hypothesis is rooted in the fact that the reunion of sponge cells does not occur in the absence of either ion (15), and recently Zeidman (18) reported that the absence of calcium or magnesium or both decreases the cohesiveness of human squamous epithelium.

The present work was designed to determine whether calcium, alone or in the presence of magnesium, will diminish or prevent the reduction in cohesiveness of the gastric epithelium induced by the mucigogue, eugenol.

PROCEDURE

The study was conducted on five Heidenhain pouch dogs, using the technique previously described (9). One per cent eugenol emulsion was made in distilled water, or in $CaCl_2$ and $MgCl_2$ solutions at the several concentrations indicated in Table 1. Tergitol-Penetrant (1/40 per cent) was used to stabilize the emulsions, as heretofore. Since an acid pH also decreases the cohesiveness of cells (3), all the eugenol emulsions were buffered with $NaHCO_3$ (about 0.1 per cent in final concentration). Determinations of pH of the emulsion before and after its application to the pouch revealed no significant deviation from neutrality in the course of an experiment. Following addition of the bicarbonate to the $CaCl_2$ solutions of highest concentrations, there was a slow precipitation of $CaCO_3$, but

* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U.S. Public Health Service.

† A preliminary report of this work was presented before the American Physiological Society at its recent meeting in Detroit. An abstract was published in Fed. Proc., 8:76, 1949.

this was barely perceptible within the first 15 minutes. Hence, the salt-containing eugenol emulsion was not prepared until immediately before administration to the pouch. Each test experiment was preceded or followed on the same day by a control experiment with eugenol emulsion in distilled water.

Volume, viscosity, and opacity of half-hour samples of mucus were recorded over a period of 2 hours following each application of stimulus. Viscosity and opacity were evaluated on a scale from 1 to 5, using the standards described by Sober *et al.* (17). High viscosity and opacity are generally considered indicative of high columnar cell content, since these three factors are statistically correlated in significant degree (9). However, the presence of such cells was confirmed by microscopic examination of smears stained with toluidine blue. This was considered essential because coagulated mucin may also contribute to the opacity of mucus in considerable measure.

TABLE 1

CHARACTERISTICS OF GASTRIC MUCUS SECRETED IN RESPONSE TO 1 PER CENT AQUEOUS EUGENOL EMULSIONS CONTAINING ABOUT 0.1 PER CENT $NaHCO_3$ AND OTHER SALTS

Eugenol emulsion made in:	No. of expts.	No. of samples	Average viscosity	Average opacity	Average volume	Columnar Cell content
Dist. H_2O	12	45	3.4	2.8	5.6	Considerable
0.02% $CaCl_2$	5	18	3.1	3.1	5.6	"
0.04% $CaCl_2$	3	10	4.6	2.6	2.6	"
0.06% $CaCl_2$	2	8	3.8	2.4	5.3	"
0.01% $MgCl_2$, 0.02% $CaCl_2$	2	8	3.2	2.9	6.4	"
0.03% $MgCl_2$, 0.06% $CaCl_2$	2	8	4.4	3.4	4.1	"

RESULTS

At none of the ionic concentrations here employed did the salts significantly affect the viscosity, opacity, and columnar cell content of the mucus produced by topical application of eugenol emulsion. The mean viscosity and opacity values (Table 1) are not appreciably different from those for the control experiments, and the smears invariably demonstrate the presence of a high columnar cell content.

DISCUSSION

The desquamating action of 1 per cent aqueous eugenol emulsion is not prevented by adding calcium, alone or with magnesium, to the emulsion. This observation is in accord with the work of Zeidman (18) in that he was unable to reverse the decreased cohesiveness of human squamous epithelium, induced by a calcium-free solution, by restoring the ion to the medium. On the other hand, Chambers (4) found that the diminished cohesiveness of capillary endothelium, induced by a calcium-free perfusate, is reversed by changing to normal Ringer's solution.

Our finding may be interpreted in any one of three ways: (1) the reaction between eugenol and the cement substance may involve calcium and magnesium in an irreversible (possibly non-ionic) manner, so that the presence of these cations at the surface of action of the desquamatory agent exercises no effect on this chemical process; or (2) this chemical reaction may be reversible, but eugenol is so powerful a desquamatory agent that its mass law effect cannot be offset by even the highest concentrations of these alkaline earth ions, which can be maintained at the surface of the mucosa under the conditions of these experiments; or (3) the desquamatory action of eugenol may be entirely independent of calcium and magnesium, *i.e.*, the mucigogue may act on some part of the cement substance which does not contain these elements. Apropos of the second of these possible interpretations, it should be noted that the concentration of eugenol used in these experiments was only 1 per cent, rather than 5 per cent as is being employed in most of our other studies with this mucigogue.

The calcium deficiency found in malignant tissues, and which has been associated with their ability to invade adjacent tissues, may reflect some alteration of a calcium-binding complex in the cement substance (12). The chemical character of this complex is uncertain, but a protein structure has been suggested (12), and it may even be a lipoprotein (7). The desquamating ability of eugenol might be attributed to its lipid solvent ability, but as yet this does not seem feasible because hypertonic NaCl likewise produces desquamation in considerable degree (9). Although there is good reason to believe that mucigogue action is not necessarily accompanied by desquamatory action, the problem of how to stimulate large quantities of a cell-free gastric mucous secretion still remains unsolved.

SUMMARY

1. The object of these experiments was to determine whether calcium and magnesium can reduce eugenol-induced desquamation of gastric columnar epithelium. This was studied in dogs by the topical

application to Heidenhain pouches of 1 per cent buffered ($NaHCO_3$) eugenol emulsions containing these ions at several different concentration levels.

2. Such eugenol emulsions exercise the same mucigogue and desquamatory actions as do control emulsions containing none of the added electrolytes.

3. This finding fails to give support to the idea of a possible relation between the desquamatory action of a gastric mucigogue and the process of invasion by cancerous tissue.

REFERENCES

1. Brunschwig, A., Dunham, L., and Nichols, S. Potassium and Calcium Content of Gastric Carcinoma. Cancer Research, **6**:230–232, 1946.
2. Carruthers, C., and Suntzeff, V. The Role of Calcium in Carcinogenesis. Science, **99**:245–247, 1944.
3. Chambers, R., and Zweifach, B. W. Capillary Endothelial Cement. J. Cell. and Comp. Physiol., **15**:255–272, 1940.
4. Chambers, R. The Relation of Extraneous Coats to Organization and Permeability of Cellular Membranes. Cold Spring Harbor Symposia, **8**:144–153, 1940.
5. Coman, D. R. Decreased Mutual Adhesiveness, a Property of Cells from Squamous Cell Carcinomas. Cancer Research, **4**:625–629, 1944.
6. Coman, D. R. Mechanism of the Invasiveness of Cancer. Science, **105**:347–348, 1947.
7. Coman, D. R. Personal Communication.
8. Herbst, C. Über das Auseinandergehen von Furchungs- und Gewebezellen in kalkfreiem Medium. Arch. f. Entwcklngsmechn. d. Organ., **9**:424–463, 1900.
9. Hollander, F., Stein, J., and Lauber, F. U. The Consistency, Opacity, and Columnar Cell Content of Gastric Mucus Secreted under the Influence of Several Mild Irritants. Gastroenterology, **6**:576–595, 1946.
10. Hollander, F., Lauber, F. U., and Stein, J. Some Characteristics of Gastric Secretion Induced by Mustard Oil Suspension. Am. J. Physiol., **149**:724–731, 1947.
11. Hollander, F., and Lauber, F. U. Eugenol as a Stimulus for Gastric Mucous Secretion. Proc. Soc. Exper. Biol. and Med., **67**:34–37, 1948.
12. Lansing, A. I. Calcium and Growth in Aging and Cancer. Science, **106**:187–188, 1947.
13. Lillie, R. S. The Relation of Ions to Contractile Processes. I. The Action of Salt Solutions on the Ciliated Epithelium of *Mytilus edulis*. Am. J. Physiol., **17**:89–141, 1906.
14. Overton, E. Beiträge zur allgemeinen Muskel- und Nervenphysiologie. III. Studien über die Wirkung der Alkali- und Erdalkalisalze auf Skelettmuskeln und Nerven. Arch. f. d. ges. Physiol. (Pflügers), **105**:176–290, 1904.
15. Robertson, J. D. The Function and Metabolism of Calcium in the Invertebrata. Biol. Rev., **16**:106–133, 1941.
16. Scott, G. H. Mineral Distribution in the Cytoplasm. Biol. Symposia, **10**:277–289, 1943.
17. Sober, H. A., Hollander, F., and Sonnenblick, B. P. Response of the Gastric Mucous Barrier in Pouch Dogs to Repeated Topical Application of Eugenol. In preparation.
18. Zeidman, I. Chemical Factors in the Mutual Adhesiveness of Epithelial Cells. Cancer Research, **7**:386–389, 1947.

Adenocarcinoma in the Uterus of an Endocrine Imbalance Female Rat*

CARROLL A. PFEIFFER, PH.D.

(From the Department of Anatomy, Yale University School of Medicine, New Haven 11, Connecticut)

In a study of the development of leiomyomas in female rats with an endocrine imbalance (3) a malignant tumor which had many metastases was found in one of the animals. This tumor was identified as a uterine adenocarcinoma. Since such a tumor had not previously been seen in our colony, it was thought that the endocrine imbalance might have been a contributing factor. Bullock and Curtis (1) have reported four such tumors among 2450 rats with cysticercus tumors and 489 rats with spontaneous tumors.

MATERIAL AND METHODS

The testes from a littermate were grafted into the cervical region of this rat within a few hours after birth using a technique previously described (3). After weaning, the animal was kept on a Purina Dog Chow diet. All tissues were preserved in Bouin's fluid, imbedded in paraffin, sectioned at 7 μ and stained with Harris' hematoxylin and triosin. A few slides were mordanted in Zenker's fluid and stained with Mallory's or Masson's trichrome stains.

DESCRIPTION

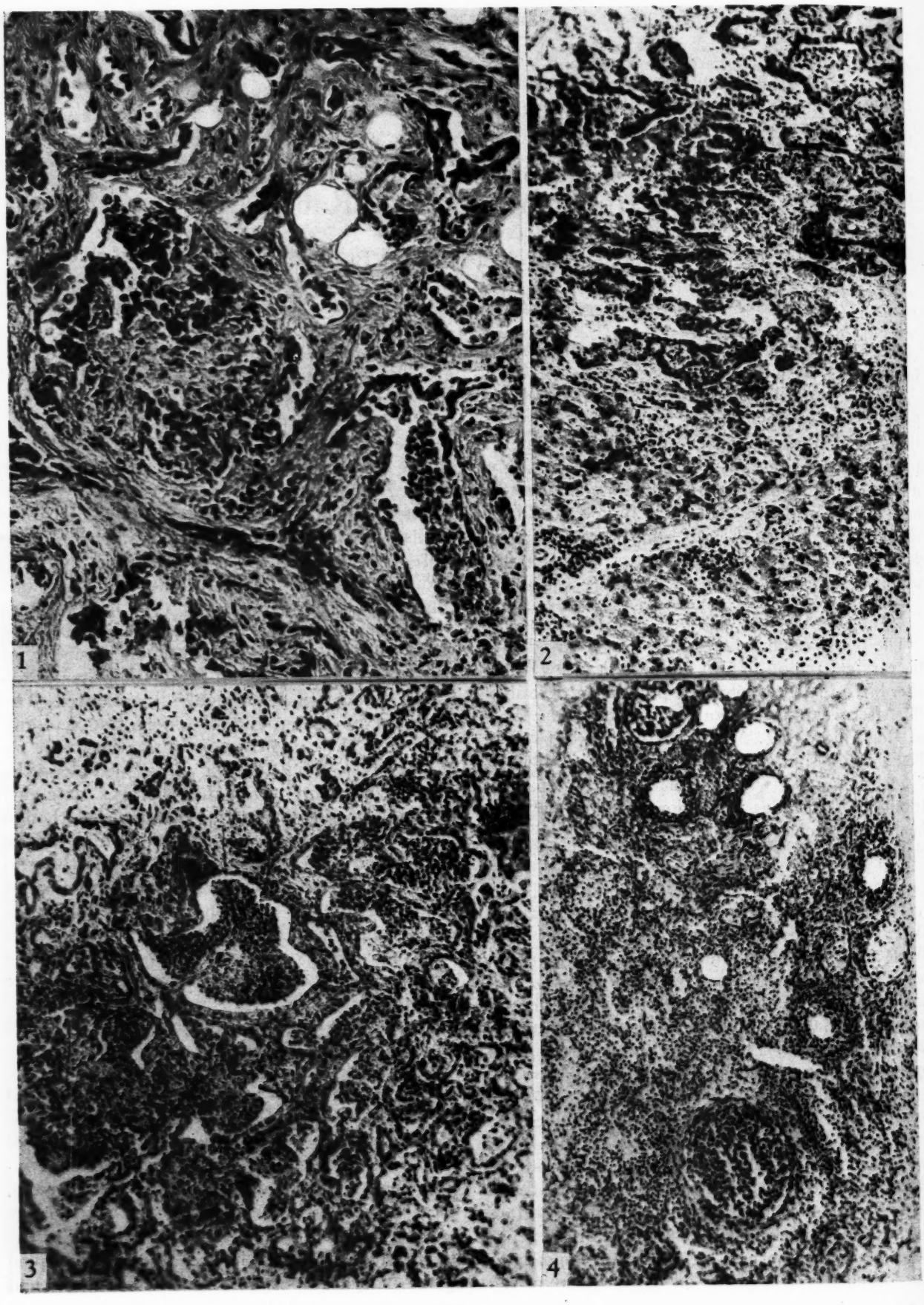
This animal began to show continuous estrus in the vaginal smears upon reaching sexual maturity. While vaginal smears were not followed throughout life, it could be assumed, on the basis of records on numerous other endocrine imbalance female rats, that such a condition existed until age factors interfered. There was no outward sign of pyometra. The rat died at the age of 816 days.

At autopsy a mass measuring 3.5 × 2.5 cm. was found in the left uterine horn. It involved the ovarian region and extended almost to the cervix posteriorly. The ovary and oviduct could not be identified by dissection since the tumor had also involved the peritoneum and fat in this region. There were several large masses, more or less separated from each other, in the lesser omentum. They filled the area of the lesser curvature of the stomach and extended up along the lower esophagus. The pancreas could not be identified. Some of these masses measured 1 × 1 cm. and were definitely metastases to the lymph nodes in the gastrosplenic ligament. The lymph nodes in other parts of the body were normal in size. There was a marked congestion of the lungs. The liver and spleen had light colored areas that appeared to be metastases to these organs. A tapeworm was also present in the liver. There was extreme hydronephrosis on the left side. The pituitary gland was enlarged and hemorrhagic.

Because the tumor involved so much tissue around the gastroduodenal area, the possibility of the stomach or duodenum being the primary site of the tumor was thoroughly investigated. The stomach, duodenum and all adnexa that had any relation to the tumor masses in this area were removed *in toto*. The stomach was opened along the greater curvature, and the slit was extended through the pyloris and the duodenum. Although the mucosa appeared normal and complete, the areas where the tumor seemed to invade the wall of the stomach and duodenum were sectioned. It was found that the tumor had invaded the serosa and in some places the muscle layers, but the mucosa and submucosa were uninvolved, indicating that the tumor did not arise from the digestive tract. From a study of a slide of the tumor alone it could easily have been mistaken for an adenocarcinoma of the stomach.

The tumor, a typical adenocarcinoma (Fig. 1), was diagnosed as having arisen in the left horn of the uterus. Mitotic figures were fairly numerous, and the tumor as a whole gave the appearance of malignancy, a fact borne out by its extensive invasiveness. The tumor was negative for mucin. Tissues readily identified as belonging to the ovary and oviduct were found when that portion of the tumor which involved the area normally occupied by the ovary was sectioned serially. The tumor had apparently invaded this region by direct extension through the mesosalpinx and mesovarium, reach-

* This investigation was aided by grants from The Anna Fuller Fund and the National Cancer Institute (U.S.P.H.S.).



Figs. 1–4

ing the ovary through the ovarian stalk. It had replaced more than one-third of the ovary. The remainder of the ovary was typical of the old constant estrous rat. There were no corpora lutea present and only a few small follicles (Fig. 4), most of which contained small amounts of follicular fluid. The stroma of the ovary was dense, and the interstitial cells resembled those of a hypophysectomized animal. Both the ovarian capsule and the surrounding tissue were invaded. The oviduct could still be identified. It exhibited a marked fibrosis of the deeper portion of the submucosa. The right uterine cornu showed a thickening of the submucosa due to an increase in collagenous fibers, particularly in the deeper portion of the endometrium.

The lymph nodes in the gastrosplenic ligament and along the lesser curvature of the stomach were completely replaced by tumor tissue (Fig. 3) which had grown to form the large discrete bodies seen in the gross dissection. No lymphoid tissue remained in these bodies. In histological structure they could not be distinguished from the primary tumor. Some of the nodes in the lesser curvature of the stomach had coalesced after being invaded by the tumor and in turn had invaded the serosa of the stomach and esophagus, the liver (Fig. 2) and probably the lungs by direct extension. The pancreas was almost completely replaced by the tumor, and the spleen had been extensively invaded. The left kidney showed the typical picture of hydronephrosis. The adrenals were slightly hypertrophied, due primarily to an enlargement of the medulla. There were, however, a few hyperplastic nodules of cortical tissue. The adrenal changes in endocrine imbalance rats will be described elsewhere. The hypophysis was not prepared for histological study due to postmortem changes.

DISCUSSION

It is, of course, impossible to say whether the endocrine imbalance in this rat was responsible for the adenocarcinoma of the uterus present, but it would seem logical that it may have played a definite role since it caused the uterus to be continuously under the influence of estrogen, even though the estrogen was present at a relatively low level (2). Whether or not there was a relationship between the endocrine imbalance and the production of the tumor, this type of tumor is so rare in the rat (1) that it would seem worth reporting.

While it must be concluded from the involvement of the peritoneum and body wall that the tumor had to some degree metastasized by direct extension, it is thought, because of the marked replacement of lymph nodes by tumor tissue, that the principal route of metastases was through the lymphatics. However, only the mesenteric nodes seemed to be involved. It seems probable that the hydronephrosis on the left side was related to the pressure exerted by the tumor, although it is possible that there was no relationship between these two conditions. The adrenal changes in this animal were presumably related to the endocrine imbalance since they have also been seen in a great number of old endocrine imbalance female rats, which will be described and discussed elsewhere.

SUMMARY

An adenocarcinoma of the uterus was found in an endocrine imbalance female rat which died at 816 days of age. The endocrine imbalance was produced by the presence of a testis graft received at birth, and consisted of a constant relationship between gonadotrophic hormone and the ovary such that estrogen was released at a constant level throughout the life of the rat. The tumor involved the entire left horn of the uterus and the ovary. It had invaded the peritoneum and had been carried through the lymphatics to the region of the lesser curvature of the stomach where it involved the pancreas, liver, spleen, serosa of the stomach and esophagus.

REFERENCES

1. BULLOCK, F. D., and CURTIS, M. R. Spontaneous Tumors of the Rat. J. Cancer Research, **14**:1–115, 1930.
2. PFEIFFER, C. A. Sexual Differences of the Hypophyses and Their Determination by the Gonads. Am. J. Anat., **58**:195–225, 1936.
3. PFEIFFER, C. A. Development of Leiomyomas in Female Rats with an Endocrine Imbalance. Cancer Research, **9**: 277–281, 1949.

FIGS. 1–4.—All tissues were sectioned at 8 μ and stained with Harris' hematoxylin and triosin.

FIG. 1.—The histological character of the adenocarcinoma of the uterus. ×220.

FIG. 2.—Invasion of the liver by the uterine adenocarcinoma. ×110.

FIG. 3.—Metastasis of the uterine adenocarcinoma to a lymph node, showing the complete replacement of the lymphoid tissue. ×110.

FIG. 4.—A small portion of the left ovary that could be identified in the tumorous mass which involved the entire area around the ovary. ×100.

Amino Acids in Epidermal Carcinogenesis in Mice*†

EUGENE ROBERTS, A. LEE CALDWELL, G. H. A. CLOWES, V. SUNTZEFF, C. CARRUTHERS, AND E. V. COWDRY

(From the Division of Cancer Research, Department of Anatomy, Washington University School of Medicine, St. Louis 10, Missouri, and The Lilly Research Laboratories, Indianapolis, Indiana)

The present investigation is one of a series dealing with the nitrogen metabolism of mouse epidermis in various phases of growth and in carcinogenesis (1 to 4). The content of 12 amino acids was determined in dried ground samples of normal epidermis and epidermis of mice receiving varying numbers of standardized applications of methylcholanthrene (MC) in benzene (0.6 gm. in 88 gm. of benzene) or of benzene alone. A transplantable squamous cell carcinoma (Tumor I), originally derived from a carcinoma produced on the back of a mouse by the application of MC (5), was also studied.

MATERIALS AND METHODS

The methods employed for the treatment of the animals and for the preparation of the tissues have been described previously (6). The pooled tissues were dried rapidly to constant weight *in vacuo* over P_2O_5 prior to hydrolysis and the water content calculated. Five individual pooled samples of normal epidermis were analyzed, four tumor samples, two samples each of tissues receiving 1, 6, 12, and 24 paintings of the carcinogen, and one sample of all of the other tissues reported. Material obtained from approximately 1500 mice was used in these studies. Nitrogen determinations were performed in duplicate or triplicate by a micro-Kjeldahl procedure.

The animo acids were determined in hydrolysates of the whole tissue. Acid hydrolysis was employed for all of the amino acids except tryptophan, for the determinations of which an enzymatic digest was made with pancreatin. Leucine, valine, isoleucine, cystine, methionine, lysine, phenylalanine, histidine, arginine, and threonine were determined by adaptations of the techniques of Barton-Wright (7). Glutamic acid was assayed according to the method of Hac, *et al.* (8), but the samples were prepared for analysis as outlined by Barton-Wright (7). The tryptophan content was measured according to Greene and Black (9), but the basal medium was modified to conform in part to that of the U.S.P. XIII nicotinic acid test.

* Aided by grants from the U.S. Public Health Service and The Charles F. Kettering Foundation.

† Presented in part before the Fourth International Cancer Research Congress held in St. Louis, Mo., Sept. 2–7, 1947.

The results are expressed in mg. of amino acid per 100 mg. of dry weight of tissue or mg. of amino acid nitrogen per 100 mg. of tissue nitrogen. The latter method is preferable since variations in non-nitrogenous constituents such as fat or glycogen can affect the former calculation. In general, the results showed the same trends by either method of calculation. Comparisons of the values for the various amino acids expressed on the total nitrogen basis among the different groups of animals were made by the use of analysis of variance (10). There were no statistically significant correlations of the number of treatments with the degree of change in the constituents studied. It was, therefore, possible to combine the values for the tissues receiving different numbers of paintings with pure benzene or with the carcinogen in single means. The values for the epidermis of mice receiving 3 paintings with MC are also listed separately because the changes after this treatment seemed more marked than in the other carcinogen-treated groups.

DISCUSSION OF RESULTS

Water, nitrogen, and total amino acid contents.—The water content of the epidermis increased on treatment with the carcinogen and showed a further increase in the carcinoma, in confirmation of previous work (11) (Table 1). On the other hand, benzene alone produced definite decreases after 12 and 24 paintings.

There was an increase in the nitrogen content of the tissues after treatment with MC when the results were expressed in percentage of dry weight, and a return to normal in the tumors. There was a slight decrease in the benzene-treated tissues. These changes in the N:weight ratio are for the most part accounted for by altered lipid contents.[1]

The sums of the 12 amino acids showed highly significant increases as a result of the treatment with MC and in the tumors by either method of calculation. If it can be assumed that the undetermined amino acids showed a trend similar to the 12 that were determined, the data can be taken to indicate a decrease in non-amino acid nitrogen relative to amino acid nitrogen in the MC-treated epi-

[1] Unpublished findings.

dermis and in the tumors. This is supported by the decreases in urea and ammonia which were found previously (4). Benzene treatment produced a smaller relative increase in amino acid nitrogen.

Changes in amino acids produced by the application of benzene.—The levels of lysine, isoleucine, leucine, phenylalanine, threonine, histidine, and glutamic acid were not significantly altered by painting with pure benzene ($P > 0.05$) (Table 2). The arginine, tryptophan, and valine contents were elevated ($0.01 < P < 0.05$). Cystine was higher in the epidermis of the treated animals than in the normal controls ($P < 0.01$), and the methionine was significantly lower ($P < 0.01$). The influence of benzene on the sulfur amino acids is of special interest. Two of the ways in which benzene is known to be detoxified by some species are as the *l*-phenylmercapturic acid, and following oxidation to phenolic derivatives, as phenylsulfuric acids (12). The sulfur-containing residues of these derivatives must come from cystine or methionine or both. The disturbance in the methionine and cystine levels of the epidermis after application of benzene suggests that the epidermis might perform detoxications similar to those ascribed to the liver. In this connection it is also interesting that the taurine level of epidermis is decreased by the application of benzene (3).

Changes in amino acids produced by the application of MC in benzene.—The contents of arginine ($P < 0.01$), valine ($0.01 < P < 0.05$), and cystine ($P < 0.01$) were higher in the epidermis of this group of animals than in the untreated controls, but the values were not significantly different from those of the benzene-painted mice. On the other hand, the methionine content increased over the normal value ($0.01 < P < 0.05$) after treatment with the carcinogen, as contrasted with the decrease in this amino acid produced by benzene alone, the difference between the means of the solvent-treated and MC-treated groups being highly significant ($P < 0.01$). The tryptophan content was higher than in the normal animals and lower than in those treated with benzene, but the differences were not within the 5 per cent range of significance. The values for histidine, isoleucine, phenylalanine, leucine, and lysine were higher after MC-application than either in the normal or in the benzene-painted mice, all the P-values being below 0.05. It is evident from the above discussion that the changes in the amino acid content of epidermis produced by the application of a benzene solution of methylcholanthrene are different from those produced by the solvent alone.

Amino acid pattern in the transplantable squamous cell carcinoma.—The levels for the leucine, isoleucine, lysine, methionine, valine, phenylalanine, and threonine in the tumors were significantly higher than in the epidermis of the untreated or benzene-painted mice, the P-values being less than 0.01 for all comparisons except for the threonine content in the solvent-treated mice, in which case the P-value was between 0.01 and 0.05. However, when the mean values for the above 7 amino acids in the carcinomas were compared with the means in the epidermis from MC-painted mice, only the lysine, leucine, and valine contents were found to be higher in the tumors ($0.01 < P < 0.05$). The levels of all seven of these amino acids in the epidermis of mice painted thrice with the carcinogen were very similar to those for the carcinomata. The tumors showed an elevation of tryptophan over normal epidermis ($0.01 < P < 0.05$), but similar or greater increases were found in epidermis receiving 6, 12, and 24 applications of benzene and 1 and 18 treatments with MC. The cystine and arginine contents of the carcinomata were lower than in the benzene- or carcinogen-treated series ($0.01 < P < 0.05$), but were virtually identical with the values found in normal epidermis. The glutamic acid and histidine contents of the tumors were not significantly different from those of the other groups.

GENERAL COMMENT

The chief purpose of the present investigation was to determine whether a change occurs in the over-all amino acid distribution when normal epidermis is transformed to a malignant tumor. The comparison in answering this question must be made not only between the normal epidermis and the tumor but also between the tumor and the precancerous hyperplastic tissue. Although the latter tissue, from foci in which tumors eventually arise, frequently differs markedly from normal epidermis with respect to the variables studied (13), it possesses none of the characteristics of malignancy. In previously reported work on nitrogen metabolism in epidermal carcinogenesis (4) it was found that of a series of tissues painted different numbers of times with MC, the epidermis receiving 3 applications was more like the tumors with respect to arginase activity and the percentage of total nitrogen found in the trichloroacetic acid-soluble fraction than samples of epidermis receiving greater numbers of paintings of the carcinogen. The latter findings, together with an examination of the weight changes of the tissues (4), suggested that the rate of protein synthesis might be greater in epidermis as a whole after 3 paintings than after subsequent applications of the MC. It is interesting that in the present study the thrice-painted

TABLE 1

Water, Nitrogen, and Total Amino Acid Contents

Tissue	Water Content: Per cent of fresh weight	Nitrogen Content: Per cent of dry weight		Amino Acids Determined: Per cent of dry weight		Amino Acids Determined: N as per cent of total N	
		MC§	Benzene	MC	Benzene	MC	Benzene	MC	Benzene
Normal*	59.1 (58.1–60.0)	11.62 (11.27–12.08)		35.58 (35.27–36.27)		44.83 (44.44–45.35)	
1 painting‡		12.26	11.27	40.78	37.42	49.34	49.91
3 paintings		12.43	11.07	49.00	38.34	55.50	50.83
6 paintings‡		13.27	11.27	46.83	36.71	50.92	46.73
12 paintings‡		12.97	10.62	43.79	34.49	49.24	46.64
18 paintings		13.33		48.35		52.50	
24 paintings‡		12.78	10.76	48.46	34.13	54.76	47.23
Tumor†	81.6 (81.2–82.0)	11.70 (11.00–12.53)		43.75 (39.37–44.55)		52.89 (47.50–53.95)	

* Five determinations.
† Four determinations.
‡ Two determinations.
§ Methylcholanthrene.

TABLE 2

Content of Individual Amino Acids

Amino Acid	Normal*: Mg. per 100 mg. dry wt.	Normal*: Mg. N per 100 mg. total N	Benzene†: Mg. per 100 mg. dry wt.	Benzene†: Mg. N per 100 mg. total N	Benzene+MC‡ (all samples): Mg. per 100 mg. dry wt.	Benzene+MC‡ (all samples): Mg. N per 100 mg. total N	Benzene+MC§ (3 paintings): Mg. per 100 mg. dry wt.	Benzene+MC§ (3 paintings): Mg. N per 100 mg. total N	Carcinomas‖: Mg. per 100 mg. dry wt.	Carcinomas‖: Mg. N per 100 mg. total N
Lysine	3.05 (2.68–3.47)	5.15 (4.25– 5.85)	2.91 (2.67–3.22)	5.08 (4.81– 5.53)	4.14 (3.25– 5.32)	6.29 (5.69– 8.21)	5.32	8.21	4.97 (4.39–5.76)	8.18 (7.10– 8.81)
Isoleucine	2.82 (2.56–2.93)	2.65 (2.44– 2.88)	2.65 (2.43– 2.96)	2.58 (2.31– 2.86)	3.91 (3.31– 4.48)	3.26 (2.98– 3.84)	4.48	3.84	4.19 (4.09–4.32)	3.83 (3.48– 4.06)
Leucine	4.44 (4.17–4.87)	4.17 (3.79– 4.58)	4.30 (3.91– 4.84)	4.19 (3.76– 4.68)	5.64 (5.38– 6.13)	4.69 (4.41– 5.26)	6.13	5.26	5.78 (5.34–6.09)	5.28 (5.19– 5.45)
Methionine	1.09 (1.04–1.13)	0.91 (0.87– 0.95)	0.86 (0.74– 1.03)	0.74 (0.65– 0.88)	1.47 (1.19– 1.78)	1.09 (0.93– 1.36)	1.78	1.36	1.48 (1.44–1.52)	1.20 (1.16– 1.24)
Valine	2.71 (2.26–2.89)	2.84 (2.56– 3.04)	2.80 (2.72– 3.00)	3.05 (2.90– 3.18)	3.48 (3.04– 3.76)	3.23 (2.92– 3.52)	3.66	3.51	3.77 (3.56–3.92)	3.86 (3.40– 4.25)
Phenylalanine	1.96 (1.92–2.03)	1.46 (1.38– 1.57)	1.80 (1.69– 1.92)	1.39 (1.29– 1.53)	2.70 (2.20– 2.94)	1.95 (1.53– 2.86)	2.94	2.00	2.60 (2.56–2.63)	1.89 (1.78– 1.98)
Threonine	2.37 (2.21–2.53)	2.43 (2.35– 2.56)	2.11 (1.90– 2.82)	2.26 (2.02– 2.95)	2.87 (2.34– 3.33)	2.62 (2.24– 3.15)	3.33	3.15	3.15 (2.95–3.86)	3.17 (2.76– 3.83)
Histidine	1.38 (1.31–1.45)	3.33 (3.20– 3.45)	1.32 (1.27– 1.48)	3.24 (3.09– 3.49)	1.70 (1.55– 1.86)	3.57 (3.43– 3.78)	1.62	3.52	1.47 (1.44–1.48)	3.40 (3.10– 3.63)
Glutamic acid	9.43 (9.02–9.72)	7.90 (7.13– 8.64)	9.12 (7.70–10.03)	7.86 (6.80– 8.46)	11.65 (9.64–12.48)	8.65 (7.49– 9.53)	12.42	9.53	9.47 (8.81–9.82)	7.72 (7.20– 8.20)
Cystine	1.47 (1.22–1.73)	1.51 (1.22– 1.78)	2.24 (1.85– 2.72)	2.38 (1.92– 2.86)	2.27 (1.94– 2.91)	2.06 (1.80– 2.64)	2.12	1.98	1.42 (1.34–1.48)	1.42 (1.25– 1.56)
Arginine	4.06 (3.43–4.32)	11.50 (9.70–12.70)	4.72 (4.06– 5.66)	13.79 (11.85–16.15)	5.35 (4.48– 5.91)	13.53 (12.34–14.29)	4.48	12.34	4.10 (3.33–4.63)	11.34 (8.55–13.05)
Tryptophan	0.82 (0.62–1.16)	0.98 (0.75– 1.50)	1.36 (0.82– 1.86)	1.71 (1.05– 2.36)	1.02 (0.72– 1.54)	1.10 (0.78– 1.73)	0.72	0.80	1.35 (1.17–1.73)	1.60 (1.31– 2.16)

* Five samples.
† Five samples.
‡ Ten samples.
§ One sample.
‖ Four samples.

epidermis showed an excellent agreement with the carcinoma in the content of lysine, isoleucine, leucine, methionine, valine, phenylalanine, threonine, and histidine. The increases in cystine and arginine found in the carcinogen-treated epidermis may have been a result of the effect of the solvent alone, since the benzene-treated tissues showed similar increases. This effect did not carry over to the tumors, which had values close to those found in normal epidermis for these amino acids.

It appears clear from the results that the changes in the distribution of amino acids produced by benzene alone are different from those taking place in carcinogenesis. However, it cannot be said that the tumor tissue as a whole shows a distinctive pattern of amino acids which would set it completely apart from all of the non-malignant hyperplastic epidermal samples.

Changes which take place in the total amino acid content of tissues, such as those reported herein, may result from alterations in the ratios of cell types and quantitative and qualitative changes in intracellular constituents. The characterization of various cell fractions with respect to amino acid content is now under way in this laboratory in an effort to determine the physiological significance of the changes reported in this paper. It is already evident that the changes found in the total amino acids cannot be directly correlated with the changes in free amino acids, since it has been previously shown (3) that there is a general increase in the individual free amino acids over the normal in the hyperplastic epidermis and a marked decrease in the carcinomata.

SUMMARY

1. The contents of lysine, isoleucine, leucine, methionine, valine, phenylalanine, threonine, histidine, glutamic acid, cystine, arginine, and tryptophan were determined by microbiological methods in hydrolysates of dried ground samples of whole normal mouse epidermis, of epidermis of mice receiving varying numbers of standardized applications of methylcholanthrene (MC) in benzene or of benzene alone, and of transplantable squamous cell carcinomata originally derived from a carcinoma produced on the back of a mouse by the application of the carcinogen.

2. The sum of the 12 amino acids showed highly significant increases over normal as a result of the treatment with MC and in the tumors when the results were expressed either in terms of mg. of amino acids per 100 mg. of dry weight of tissue or mg. of amino acid nitrogen per 100 mg. of tissue nitrogen. Benzene treatment produced a smaller relative increase in amino acid nitrogen and no significant change on the dry weight basis.

3. From a consideration of the individual values for the amino acids in the tissues studied it was concluded that benzene alone produced significant changes in amino acid distribution in the epidermis and that more extensive and different alterations were produced when MC in benzene was applied. The distribution of amino acids in the carcinomata was similar in most respects to that found in the precancerous hyperplastic epidermis.

REFERENCES

1. ROBERTS, E., and FRANKEL, S. Urea and Ammonia Content of Mouse Epidermis. Arch. Biochem., **20**:386–393, 1949.
2. ROBERTS, E. Estimation of Arginase Activity in Homogenates. J. Biol. Chem., **176**:213–222, 1948.
3. ROBERTS, E., and TISHKOFF, G. H. Distribution of Free Amino Acids in Mouse Epidermis in Various Phases of Growth as Determined by Paper Partition Chromatography. Science, **109**:14–16, 1949.
4. ROBERTS, E., and FRANKEL, S. Arginase Activity and Nitrogen Content in Epidermal Carcinogenesis. Cancer Research, **9**:231–237, 1949.
5. COOPER, Z. K., FIRMINGER, H. I., and RELLER, H. C. Transplantable Methylcholanthrene Skin Carcinomas of Mice. Cancer Research, 1944. **4**:617–621,
6. BAUMBERGER, J. P., SUNTZEFF, V., and COWDRY, E. V. Methods for the Separation of Epidermis from Dermis and Some Physiologic and Chemical Properties of Isolated Epidermis. J. Nat. Cancer Inst., **2**:413–423, 1942.
7. BARTON-WRIGHT, E. C. Microbiological Assay of Amino Acids. I. Tryptophan, Leucine, Isoleucine, Valine, Cystine, Methionine, Lysine, Phenylalanine, Histidine, Arginine, Threonine. Analyst, **71**:267–278, 1946.
8. HAC, L. R., SNELL, E. E., and WILLIAMS, R. J. The Microbiological Determination of Amino Acids. II. Assay and Utilization of Glutamic Acid and Glutamine by Lactobocillus Arabinosus. J. Biol. Chem., **159**:273–289, 1945.
9. GREENE, R. D., and BLACK, A. The Microbiological Assay of Tryptophan in Proteins and Foods. J. Biol. Chem., **155**:1–8, 1944.
10. SNEDECOR, G. W. Statistical Methods Applied to Experiments in Agriculture and Biology. Ames: The Iowa State College Press, 1946.
11. SUNTZEFF, V., and CARRUTHERS, C. The Water Content in the Epidermis of Mice Undergoing Carcinogenesis by Methylcholanthrene. Cancer Research, **6**:574–577, 1946.
12. WILLIAMS, R. T. Detoxication Mechanisms. New York: John Wiley & Sons, Inc., 1947.
13. COWDRY, E. V. Epidermal Carcinogenesis. J.A.M.A., **135**:408–411, 1947.

The Effect of Dietary Fat and Carbohydrate on Diethylstilbestrol-induced Mammary Cancer in Rats*

W. F. Dunning, Ph.D., M. R. Curtis, Ph.D., and M. E. Maun, M.D.

(From the Department of Pathology, Wayne University College of Medicine, Detroit Institute of Cancer Research, and St. Mary's Hospital, Detroit 1, Michigan)

Overweight in man has been correlated with a higher incidence of cancer than that observed in normal or in underweight persons (12). Considerable experimental evidence indicates that diets high in fat or in caloric content increase the probability of the occurrence of many types of cancer in the mouse (1, 2, 5, 15, 18). Conversely, restricted caloric intake (13, 14, 17) prevents or delays the onset of both experimentally induced and spontaneously occurring neoplasms in the mouse. In the rat, dietary fat (6) was found to be much less effective in enhancing chemically induced skin cancer than in the mouse. In the absence of added fat in a semi-synthetic diet (11), no liver tumors were induced by *p*-dimethylaminoazobenzene, but the rats grew poorly and few survived the minimum latent period, while the addition of varying quantities of fat proportionately accelerated the formation of these liver tumors. An exception appears to be hydrogenated cocoanut oil (8). When 5 per cent hydrogenated cocoanut oil was the only source of fat in a synthetic diet, the formation of dimethylaminoazobenzene-induced liver tumors was retarded. Chronic undernourishment (7) effectively prolonged the life span of rats and delayed the onset of pathological ageing processes and also gave limited evidence of a retardation in the development of spontaneous tumors.

In the mouse, the amount of estradiol benzoate (16) required to produce a minimum duct response of the mammary gland is considerably increased as the level of food intake is decreased. Chronic underfeeding also results in pseudo-hypophysectomy (9) and lack of adrenotropic hormone (10). Because caloric intake in mice has proved to be such an important factor in the initiation of spontaneously occurring mammary cancers, and because rats on high-fat diets tend to ingest more calories per day than on diets of low fat content, it seemed important to assay the effects of dietary fat under conditions in which the caloric intake was rigidly controlled, even though it necessitated limiting considerably the number of rats that could be used. Accordingly the following experiments were undertaken.

* Supported in part by a grant-in-aid from the United States Public Health Service.

MATERIAL AND METHODS

Pedigreed female rats of A × C Line 9935 between 4 and 5 months of age were used for the experiments. Cholesterol pellets containing 4 to 6 mg. of diethylstilbestrol[1] were implanted subcutaneously in the scapular region to maintain a continuous state of hyperestrinism. A previous report (3) showed that on the laboratory stock diet of Friskie Dog Pellets supplemented by a green vegetable once a week, 85 per cent of the rats of this line, when similarly treated, developed multiple mammary cancers between the eleventh and twenty-second months.

In the present experiments each rat was housed in an individual cage with free access to water. The daily portion of food was weighed out and presented in a food cup to which was added the daily supplement of crystalline vitamins. An attempt was made to recover and weigh all food that was spilled, but there was probably some unaccounted for waste of food by the rats on the *ad libitum* diets. The first 24 rats were pair fed. That is, the mates of the rats on the *ad libitum* low-fat diet received an isocaloric ration of high-fat diet equivalent to that consumed by the mate on the low-fat diet.

The base diet consisted of the following ingredients:

	Per Cent
Cellu flour	2.0
Halibut liver oil	0.4
Salt mixture	4.0
Crisco	5.0
Casin	30.0
Dextrin	60.0

plus the following crystalline vitamin[2] supplement per kilo:

Thiamin	4 mg.
Riboflavin	8 mg.
Pyridoxine HCl	4 mg.
Niacin	4 mg.
Calcium pantothenate	20 mg.
Choline HCl	2,000 mg.
Alpha-tocopherol	150 mg.

[1] Supplied through the courtesy of Dr. D. F. Robertson of Merck and Co., Rahway, N.J.

[2] Supplied through the courtesy of Dr. R. C. Pogge, Merck and Co., Rahway, N.J.

The base diet was combined with Crisco and dextrin to make up the three following rations:

I. Low Fat	II. Modified Low Fat	III. High Fat
30 gm. base	30 gm. base	30 gm. base
5 gm. Crisco	10 gm. Crisco	30 gm. Crisco
65 gm. dextrin	60 gm. dextrin	8.3 gm. dextrin
100 gm.	100 gm.	68.3 gm.

The low-fat diet contained 4.3 calories per gram, the modified low-fat diet, 4.6 calories per gram and the high-fat diet, 6.3 calories per gram. The three diets contained respectively, 6.5, 11, and 46 per cent Crisco and 83, 78, and 38 per cent dextrin. Each rat on *ad libitum* rations was offered daily a weighed portion equal to a little more than she had consumed the previous day. The rats on restricted diets always consumed all that was offered them. An attempt to restrict the caloric intake to 22 calories per day (65 per cent of the amount consumed by those on *ad libitum* low-fat rations) resulted in failure, due to the death of a majority of the rats within 60 days. A restriction to 25 calories per day permitted the rats to live beyond the average survival period of the rats on *ad libitum* rations, but they lost considerable weight, were irritable, and constantly in search of food.

The experiment included 84 rats in six groups as shown in Table 1. The first group of 12 rats consumed daily an average of 7.8 grams of the low-fat diet or 34 calories. Their paired mates consumed an iso-caloric portion or 5.4 grams of the high-fat diet and seemed well satisfied. The 12 rats fed the high-fat diet *ad libitum* averaged 6.4 grams or 40 calories in daily consumption. The three groups on restricted regimens consumed iso-caloric portions of high-fat, modified low-fat and low-fat diets equivalent to 25 calories per rat per day.

Each rat was weighed and inspected for mammary cancers once a week. At death, a thorough postmortem examination included a detailed description of every visible tumor, gross sectioning of all mammary glands and the inspection and weight of the liver, kidneys, adrenals, pituitary, and sex glands. Representative sections of each of these tissues and organs were preserved for microscopic examination. All cancer foci that could be identified were tabulated.

RESULTS

The results are tabulated briefly in Tables 2 and 3 and shown graphically in Charts 1, 2, and 3. All of the rats on *ad libitum* rations and the majority of the restricted rats survived for at least 6 months, the approximate minimum latent period for gross mammary cancers. The heaviest losses were among the restricted rats on the low-fat diet. These deaths occurred between the twenty-sixth and fifty-fifth days after being placed on the restricted diet and their records will not be considered further.

The rats fed *ad libitum* were heavier at death than the rats on the restricted diets but even those on the high-fat diets were not overweight. The livers were proportionately heavier in the rats on the high-fat diets than in the rats on the low-fat diets, but showed no evidence of fatty infiltration. The pituitaries were largest in two of the groups on the high-fat diet, *i.e.*, those on the restricted high-fat diet and the high-fat mates of the rats on the *ad libitum* low-fat diet. The mean pituitary weight for the rats on the *ad libitum* low-fat diet was 69 ± 15 mg. compared with 152 ± 22 mg. for their paired mates on the high-fat diet and 96 ± 20 mg. for the rats on the *ad lib* high-fat diet. On the restricted high-fat diet, the mean pituitary weight was 148 ± 24 mg. compared with 70 ± 14 mg. for rats on the restricted low-fat diet. Otherwise, there were no significant differences in the organs which were weighed as shown in Table 2. Although the rats on the restricted low-fat diet showed considerable inanition and, judging by our experience in attempting to reduce their consumption by an additional 3 calories daily, were fairly close to the starvation level, no changes were noted in their adrenals, which would indicate a depression in the physiological function of the pituitary.

TABLE 1

THE NUMBER OF RATS IN EACH GROUP, THEIR AVERAGE INITIAL BODY WEIGHT AND DAILY FOOD CONSUMPTION

Group	Number of rats	Body weight in gms.	Daily ration in gms.	Daily ration in cals.
Ab lib low-fat	12	152	7.8	34
High-fat paired mates	12	151	5.4	34
Ab lib high-fat	12	154	6.4	40
Restricted high-fat	14	139	3.9	25
Restricted 11 per cent fat	10	159	5.5	25
Restricted low-fat	24	143	5.8	25

Among the rats which survived for at least 6 months, the average survival period of those on the restricted rations was considerably prolonged, as shown graphically in Chart 3. The rats on the restricted high-fat diet and the restricted modified low-fat diet survived an average of 100 days longer than those on *ad libitum* rations, but judging from physical appearance, the rats which received the iso-caloric high-fat equivalent of the *ad libitum* low-fat consumption were in the best general health and those on the restricted low-fat diet were in the poorest physical condition.

The mammary cancer history is given in Table 3. Of the 67 rats which survived for 180 days, 58 or 87 per cent developed one or more mammary cancers. This is about the same proportion previously observed for rats of this line on the stock diet. The different groups varied somewhat. Comparing the paired mates on isocaloric high-fat and

low-fat diets, 9 of the 12 on the low-fat diet developed 34 gross tumors, while 12 mates had 52. Consideration of the cancer foci found on microscopic examination of the mammary tissue increased the number of cancer foci in the rats on the high-fat diet to twice that of their mates on the low-fat diet or 157 and 78, respectively. The gross tumors in the rats on the high-fat diet averaged 6.5 grams in weight compared with 5.0 grams for the tumors of their mates. The minimum and average latent periods were shorter for the tumors in the rats on the high-fat diet, being 153 and 288 days, respectively, compared with 175 and 304 days for the tumors in the rats on the low-fat diet. These observations appear to support the concept that fat is a mammary cancer accelerator. Inspection of Chart 2, however, reveals that four of the rats on the low-fat diet developed their first tumors somewhat earlier than their mates on the isocaloric high-fat diet and in one instance the rat on the low-fat diet had eighteen probably independent tumors, while its mate had only four. In another instance in which each member of a pair had 14 tumors, the rat on the low-fat diet developed its first tumor 7 weeks before a tumor appeared in the mate on the high-fat diet.

TABLE 2

THE NUMBER OF RATS WHICH SURVIVED FOR AT LEAST 180 DAYS, THEIR AVERAGE SURVIVAL IN DAYS, POSTMORTEM BODY WEIGHTS IN GRAMS, AND THE AVERAGE PERCENTAGE WEIGHTS OF SOME OF THE ORGANS

Group	Number of rats	Average survival in days	Average body wt.	Organ weights in per cent of body weight					
				Liver	Kidney	Adrenal	Pituitary	Ovary	Uterus
Ad lib low-fat	12	392	129	4.9	0.9	.04	.05	.06	.65
High-fat paired mates	12	392	144	6.0	1.2	.04	.10	.06	.50
Ad lib high-fat	12	383	145	6.1	1.1	.05	.07	.06	.46
Restricted high-fat	10	488	110	5.4	1.2	.05	.13	.06	.58
Restricted 11% fat	9	503	116	3.7	1.0	.04	.08	.03	.55
Restricted low-fat	12	431	96	4.8	1.4	.05	.07	.05	.66

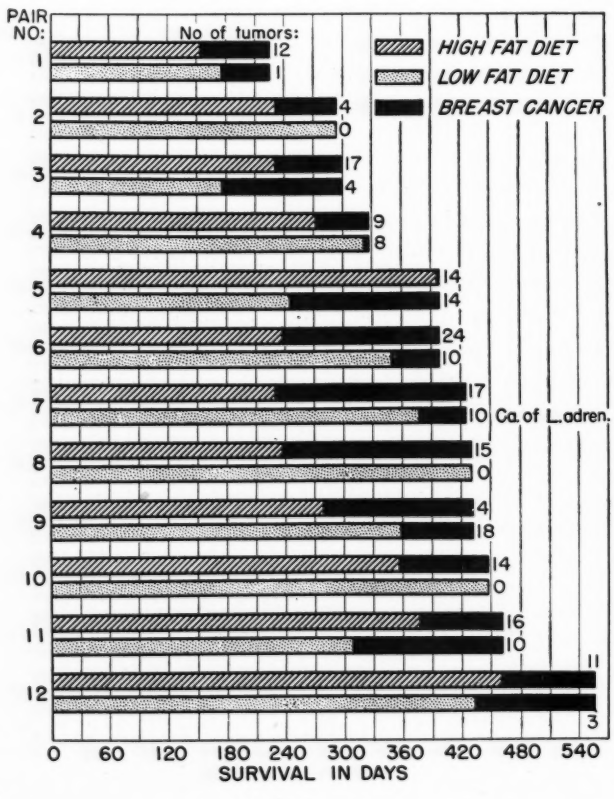


CHART 1.—Survival period and tumor history of pair-fed mates on isocaloric high- and low-fat diets. (Each rat is represented by a bar, the length of which indicates the period of survival; the blackened area represents the time elapsed after observation of the first mammary cancer, and the number at the right of the bar represents the total number of mammary cancer foci identified in the postmortem study.)

The rats on the *ad libitum* high-fat diet consumed more fat and an average of six more calories daily, but one of the twelve died without any evidence of mammary cancer and another had only two small growths. The minimum and average latent periods as shown in Chart 3 were essentially the same as for the rats on the *ad libitum* low-fat ration. One real difference seemed to be in the size of the tumors (Table 2). The average weight of the gross tumors at death was 9.3 grams or 80 per cent larger than the mean weight of 5.0 grams for the tumors in the rats on the *ad libitum* low-fat diet and more than 40 per cent higher than the average weight of 6.5 grams for the tumors in the latter's pair-fed mates on the high-fat diet. Another factor seemed to be the prevalence of tumors of the uterus in rats of this group as shown in Chart 2. Four of the 12 rats had malignant tumors involving the uterus: an adenocarcinoma, a squamous cell cancer, a mixed tumor of the ovary and uterus, and another tumor of the uterus which was probably an adenocarcinoma. No tumors of this organ were observed in the rats on the other dietary regimens, although fifty-one of them survived for more than 256 days (the number of days before the observation of the first of these tumors). Spontaneous tumors of the uterus have been observed in untreated rats of this line in

about the frequency of 1 in 200. Two additional tumors, which may be unrelated to the treatment, were a benign adenoma of the mammary gland in a rat on the restricted high-fat diet and a carcinoma of the adrenal in a rat on the *ad libitum* low-fat diet. However, adrenal tumors (4) are a fairly common sequel to estrogenic treatment in rats of another inbred line.

In general physical appearance, the 12 rats on the *ad libitum* high-fat diet compared unfavorably with the 12 rats on the same diet, limited in quantity to equal the caloric value of an *ad libitum* low-fat mate. It seemed that above a certain level additional fat consumption was detrimental to the physical well being of the rat and interfered with the initiation of the mammary cancer. These rats did not live quite as long or develop as many tumors per rat, but once a tumor could be identified, it appeared to grow much faster than the tumors of the rats in any of the other groups.

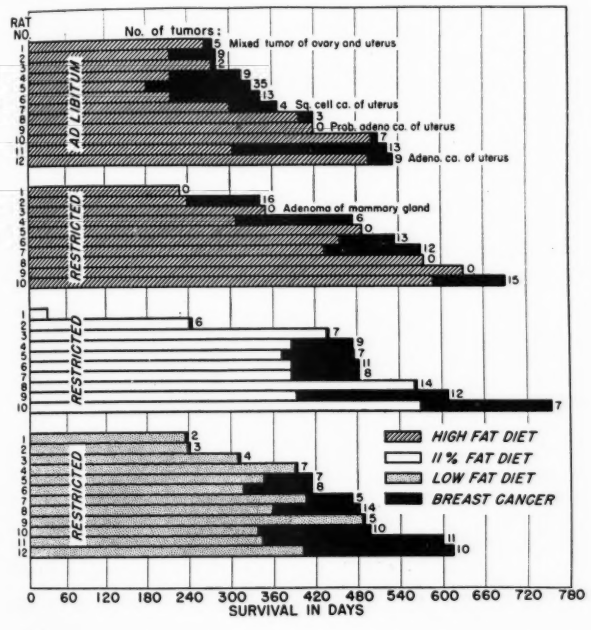


CHART 2.—Survival period and tumor history of rats on the *ad libitum* high-fat, restricted high-fat, modified low-fat, and low-fat diets. (For interpretation, see the legend for Chart 1.)

The average survival period of the rats on restricted diets was considerably prolonged and the minimum and average latent periods for the tumors were increased as shown by Chart 3. The percentage of rats which eventually developed tumors was not significantly lessened as shown in Table 3 and Chart 2. The growth of the tumors was greatly retarded as shown (Table 3) by their average weights. The number of cancer foci identified by microscopic examination of the mammary tissue was not less in the rats on the restricted low-fat diets than had been observed in the rats on the *ad libitum* low-fat diet. The percentage of rats that developed neoplasms was lowest (50 per cent) among the rats on the restricted high-fat diet, but the average number of gross and microscopic tumors per tumor-bearing rat was 12 or higher than in any other group. Probably the employment of a larger number of rats would reduce these deviations, but not alter the general observations that the state of inanition caused by a restriction in the caloric intake of the rat delays considerably the onset of the malignant process in the mammary gland, even when there is no evidence of hormonal insufficiency, and that increased fat concentration in the diet stimulates mammary growth and secretion and accelerates the growth of the once formed cancer cells.

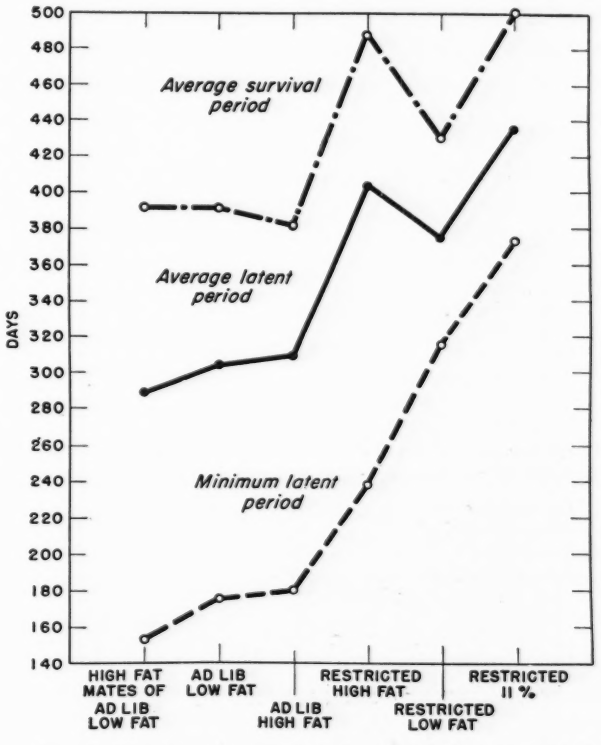


CHART 3.—The average survival period of the rats and the minimum and average latent periods of the mammary cancers in rats on each of 6 diets.

Histologically, the mammary glands from rats fed diets of high fat content showed more hypertrophy and secretory activity than did the breast tissues of the rats on diets of lower fat content. In general, more gross tumors per rat were observed and a greater number of microscopic foci were identified as neoplastic in the rats on the high-fat diets than in those of other groups. The multiple minute tumors were readily found in the breast tissue that showed the greatest hyperplasia and secretory activity.

The 236 gross tumors and 337 additional neoplastic foci found by microscopic study of the preserved mammary tissues were all classified as adenocarcinomas. The larger growths were mostly papillary and cystic. The histologic features of all of these tumors were comparable, but the development of the neoplasm could more easily be traced in the rats fed diets low in fat content because the developmental changes were less abrupt. Early alterations were generalized hyperplasia (Fig. 1) followed by an enlargement of the lobules with a coalescence of the contained follicles (Figs. 2 and 3) to form a somewhat homogenous eosin stained material. In the expanded follicles, the limits of each acinus were indistinct and the lining epithelium was partly shed. Alterations in the follicular epithelial cells were observed in the areas showing the grosser architectural changes. Cell boundaries of the very early neoplasms were sharply defined, the nuclei were larger and the nucleoli more conspicuous than in the surrounding epithelial cells. Under low power observation, the follicles of these early tumors appeared hyperchromatic (Figs. 4 and 5).

In somewhat larger tumors a sharp separation from the adjacent non-neoplastic breast stroma was evident. The central portions of the follicles had fused to form small cystic spaces (Fig. 6) and fingerlike projections from the walls of ruptured acini hung freely into these cystic spaces. The large tumors seen in the gross specimens were composed of solid masses (Fig. 7) and rounded cords of epithelial cells interspersed with cysts containing projecting masses of loose, extremely hyperchromatic cells (Fig. 8). These nodular areas showed little evidence of secretory activity and the solid cords of tumor cells simulated the appearance of early intraductal carcinoma of the human breast.

TABLE 3

THE NUMBER AND PERCENTAGE OF RATS IN EACH GROUP WITH MULTIPLE MAMMARY CANCERS, THE NUMBER AND AVERAGE WEIGHT OF THE GROSS TUMORS, THE TOTAL NUMBER OF CANCER FOCI OBSERVED, AND THE MINIMUM AND AVERAGE LATENT PERIODS IN DAYS

Group	Number of rats	Rats with ca.		Number of carcinomas		Av. weight of cas. in gms.	Minimum latent period	Average latent period
		No.	%	Gross	Total			
Ad lib low-fat	12	9	75	34	78	5.0	175	304
High-fat paired mates	12	12	100	52	157	6.5	153	288
Ad lib high-fat	12	11	92	47	109	9.3	180	309
Restricted high-fat	10	5	50	33	62	4.1	238	404
Restricted 11% fat	9	9	100	30	81	3.5	372	436
Restricted low-fat	12	12	100	40	86	4.0	316	376
Total	67	58	87	236	573			

SUMMARY

1. In order to assay the effects of dietary fat on mammary cancer development under conditions of rigidly controlled caloric intake, 84 A × C Line 9935 female rats, with cholesterol pellets containing 4 to 6 mg. of dietylstilbestrol implanted in their scapular region, were distributed into 6 groups and placed on iso-caloric synthetic rations of varying fat and carbohydrate composition.

2. Diets adequate in protein, minerals, and vitamins, but varying in fat content from 6.5 per cent to 46 per cent Crisco with sufficient dextrin to equalize the caloric content were fed *ad libitum* and restricted to rats in individual cages.

3. The caloric intake varied from 40 calories daily for rats on the *ad libitum* high-fat diet to 34 calories daily for those on the *ad libitum* low-fat diet and their paired mates on the high-fat diet, and was restricted to 25 calories daily in isocaloric portions of the high-fat, modified low-fat, and low-fat diet in three additional groups.

4. Restricting the intake to 25 calories daily permitted the rats to live well beyond the average survival periods of the rats on *ad libitum* rations, but the restricted animals lost considerable weight, were irritable and constantly in search of food.

5. Restricting the intake to 22 calories per day resulted in the deaths of the majority of the rats within 60 days.

6. Sixty-seven rats survived for at least 180 days, the approximate minimum latent period for diethylstilbestrol-induced mammary cancer in this strain, and of these, 58 or 87 per cent developed multiple mammary cancers.

7. Restricting the caloric intake to 74 per cent of the *ad libitum* consumption did not significantly decrease the percentage of rats that eventually developed mammary cancer, but increased the average latent period from approximately 300 days to 400 days.

8. A comparison of the paired mates on isocaloric high-fat and low-fat diets showed that more tumors were observed, and the average latent period was somewhat shorter in the rats on the high-fat diet than in their mates on the low-fat diet.

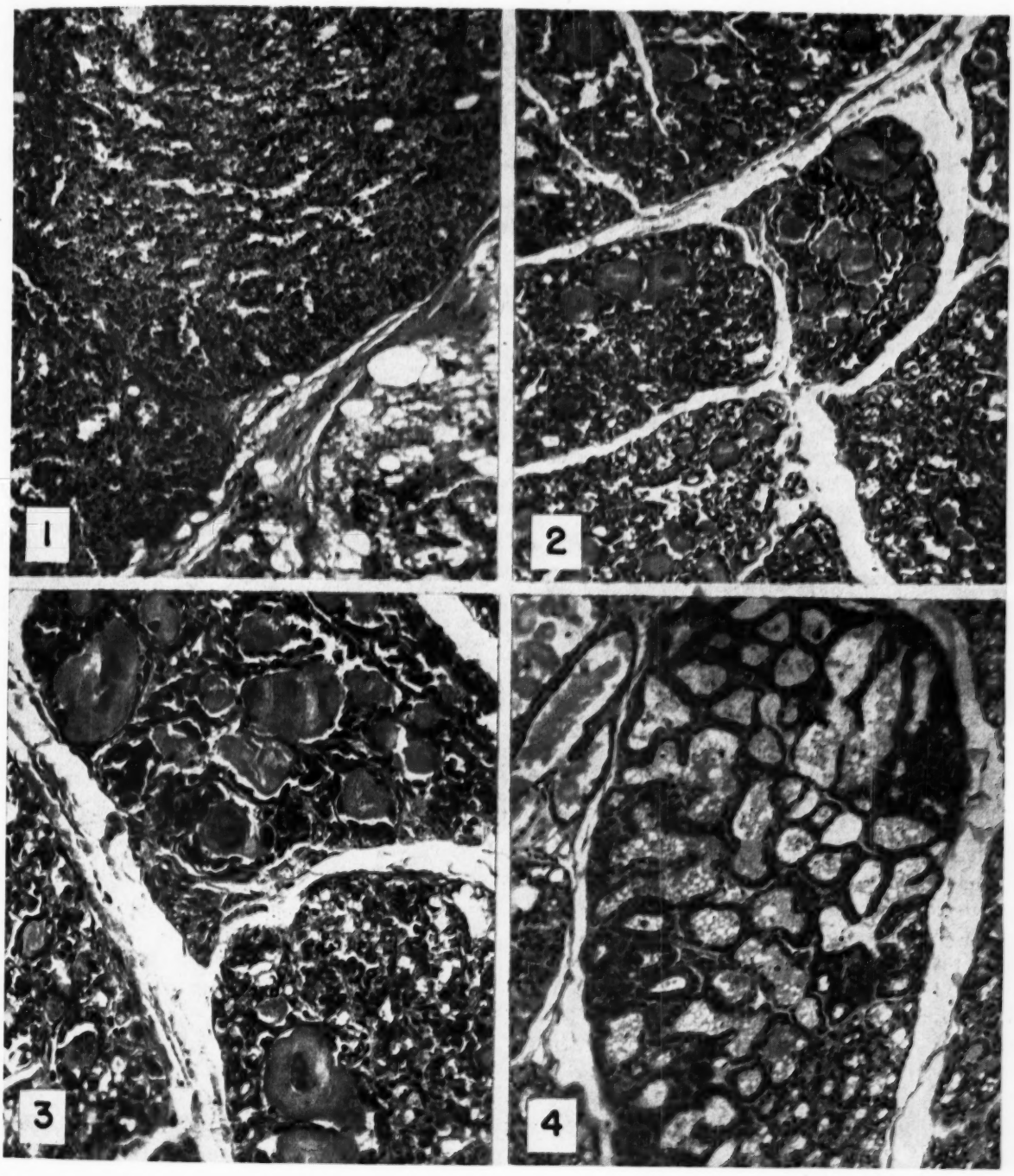


FIG. 1.—Generalized hyperplasia of the mammary gland of a rat on the high-fat diet after 327 days of treatment with a pellet containing 5 mg. of diethylstilbestrol. ×150.

FIG. 2.—Increased secretory activity and hyperchromatism in the mammary gland of a rat on the low-fat diet after 399 days of treatment with a pellet containing 5 mg. of diethylstilbestrol. ×150.

FIG. 3.—Same as Fig. 2. ×250.

FIG. 4.—Coalescent lobules and hyperchromatism in the mammary gland of a rat on the low-fat diet after 294 days of treatment with a pellet containing 5 mg. of diethylstilbestrol. ×150.

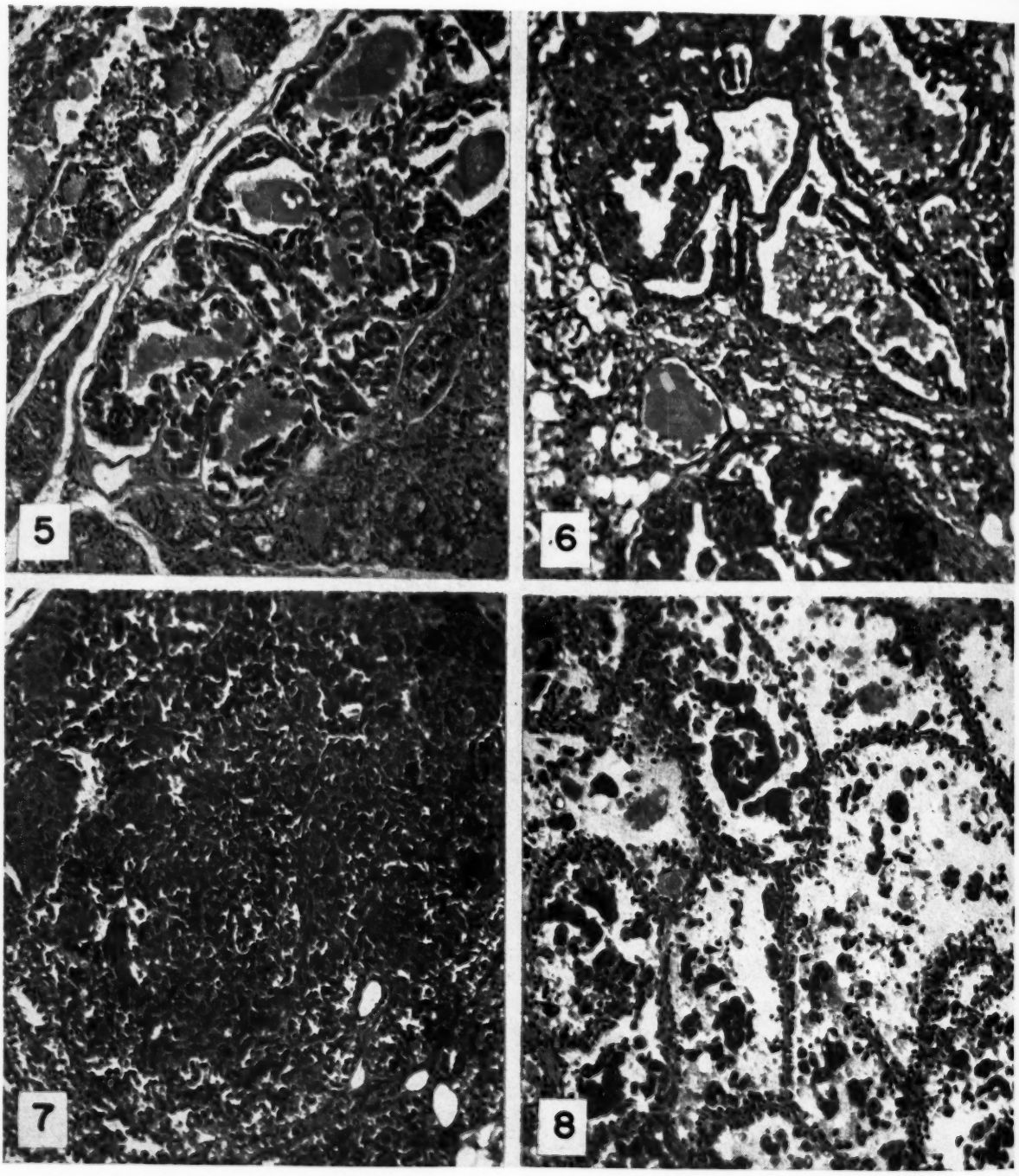


FIG. 5.—Tumor nodule in the mammary gland of a rat on the low-fat diet after 298 days of treatment with a pellet containing 5.0 mg. of diethylstilbestrol. ×150.

FIG. 6.—Multiple tumor nodules separated by normal stroma in the mammary gland of a rat on the high-fat diet after 399 days of treatment with a pellet containing 5.5 mg. of diethylstilbestrol. ×125.

FIG. 7.—Solid nodule of carcinoma in the mammary gland of a rat on the low-fat diet after 327 days of treatment with a pellet containing 5.0 mg. of diethylstilbestrol. ×150.

FIG. 8.—Malignant papillary area in the mammary gland of a rat on the low-fat diet after 399 days of treatment with a pellet containing 5.0 mg. of diethylstilbestrol. ×150.

9. When the high-fat diet was restricted to 25 calories daily, or fed *ad libitum* at an average consumption of 40 calories daily, neither the number of tumors induced nor the latent periods differed significantly from those on *ad libitum* low-fat diets.

10. The only consistent effect of the high-fat diet appeared to be an acceleration of the growth rate of the induced tumors.

11. Extensive hypertrophy and evidence of an increased secretory activity were consistent histologic features of the mammary gland of the rats on the high-fat diet, although no evidences of hormone insufficiency were observed in the mammary glands of the rats on low-fat or restricted diets.

12. All 236 gross tumors of the mammary glands and 337 additional microscopic foci were characterized as adenocarcinomas. All of the tumors were generally similar in structure, but the developmental changes appeared more abrupt in the tumors of the rats on the high-fat diet.

REFERENCES

1. BAUMANN, C. A., JACOBI, H. P., and RUSCH, H. P. The Effect of Diet on Experimental Tumor Production. Am. J. Hyg., **30**:1–6, 1939.
2. BAUMANN, C. A., and RUSCH, H. P. Effect of Diet on Tumors Induced by Ultraviolet Light. Am. J. Cancer, **35**:213–221, 1939.
3. DUNNING, W. F., CURTIS, M. R., and SEGALOFF, A. Strain Differences in Response to Diethylstilbestrol and the Induction of Mammary Gland and Bladder Cancer in the Rat. Cancer Research, **7**:511–521, 1947.
4. DUNNING, W. F., CURTIS, M. R., and SEGALOFF, A. Strain Differences in Response to Diethylstilbestrol and the Induction of Mammary Gland, Adrenal, and Bladder Cancer in the Rat. 1949, In press.
5. JACOBI, H. P., and BAUMANN, C. A. Effect of Fat on Tumor Formation. Am. J. Cancer, **39**:338–342, 1940.
6. LAVIK, P. S., and BAUMANN, C. A. Dietary Fat and Tumor Formation. Cancer Research, **1**:181–187, 1941.
7. MCCAY, C. M., ELLIS, G. H., BARNES, L., SMITH, C. A. H., and SPERLING, G. Chemical and Pathological Changes in Ageing and After Retarded Growth. J. Nutrition, **18**:15–25, 1939.
8. MILLER, J. A., KLINE, B. F., RUSCH, H. P., and BAUMANN, C. A. The Carcinogenicity of *p*-Dimethylaminoazobenzen in Diets Containing Hydrogenated Cocoanut Oil. Cancer Research, **4**:153–158, 1944.
9. MULINOS, M. G., and POMERANTZ, L. Pseudo-Hypophysectomy. J. Nutrition, **19**:493–504, 1940.
10. MULINOS, M. G., and POMERANTZ, L. Hormonal Influences on the Weight of the Adrenal in Inanition. Am. J. Physiol., **132**:368–374, 1941.
11. OPIE, E. L. The Influence of Diet on the Production of Tumors of the Liver by Butter Yellow. J. Exper. Med., **80**:219–230, 1944.
12. TANNENBAUM, A. Relationship of Body Weight to Cancer Incidence. Arch. Path., **30**:509–517, 1940.
13. TANNENBAUM, A. The Initiation and Growth of Tumors I. Effects of Underfeeding. Am. J. Cancer, **38**:335–350, 1940.
14. TANNENBAUM, A. The Genesis and Growth of Tumors II. Effects of Caloric Restriction *per se*. Cancer Research, **2**:460–467, 1942.
15. TANNENBAUM, A. The Genesis and Growth of Tumors III. Effects of a High Fat Diet. Cancer Research, **2**:468–475, 1942.
16. TRENTIN, J. J., and TURNER, C. W. Quantitative Study of the Effect of Inanition on Responsiveness of the Mammary Gland to Estrogen. Endocrinology, **29**:984–989, 1941.
17. VISSCHER, M. B., BALL, Z. B., BARNES, R. H., and SILVERTSEN, I. The Influence of Calorie Restriction upon the Incidence of Spontaneous Mammary Carcinoma in Mice. Surgery, **11**:48–55, 1942.
18. WATSON, A. F., and MELLANBY, F. Tar Cancer in Mice II. Brit. J. Exper. Path., **11**:311–322, 1930.

Beta-Glucuronidase Activity in Human Female Genital Cancer

L. D. ODELL, M.D. AND J. C. BURT, M.D.

(From the Department of Obstetrics and Gynecology, University of Chicago, and The Chicago Lying-in Hospital, Chicago 37, Illinois)

In 1937 Marrian (1) described a reaction whereby estriol glucuronide was hydrolyzed during its passage through mouse intestine. This action was ascribed to a β-glucuronidase.[1] In a series of experiments, Fishman (2) and others (3) associated the activity of this enzyme more closely with the metabolism of glucuronic acid. The glucuronidase activity of mouse liver was increased following the administration of borneal and menthol, which are conjugated in animals as glucuronides, while injections of estrogen increased the glucuronidase activity of mouse uteri. The concept of "metabolic conjugation" rather than detoxification was applied to β-glucuronidase activity in mouse uteri where it was suggested the conjugated estriol glucuronide was possibly used by the cells (4). Recently, Odell and Fishman (5) described cyclic changes of glucuronidase activity in normal human endometrium. These changes closely parallel the reported titres of blood and urinary estrogens during the menstrual cycle. In addition blood and urine glucuronidase activity were found increased during pregnancy (6, 7). Moreover, the fall in serum glucuronidase activity postpartum was delayed by the administration of estrogens, notably stilbestrol (8).

A close association with cellular activity led to the study of this enzyme in cancer tissue. Fishman (9, 10) noted greater activity of glucuronidase in breast carcinomas and involved lymph nodes, and in gastric, bowel, and metastatic carcinomas, than in normal uninvolved tissues. Moreover, an inhibitor substance (11) low in cancer tissue was detected. Preliminary studies on female genital cancer have been reported by Odell and Burt (12).

EXPERIMENTAL

The method used was that described by Fishman, Springer and Brunetti (13). Fresh tissues[2] were weighed, homogenized with distilled water, centrifuged and the supernatant fluids assayed for β-glucuronidase activity. Findings are summarized in Tables 1, 2, 3, and 4. Except for the pregnant cervix, a consistent difference in glucuronidase activity occurred between histologically proven cases of squamous celled cervical carcinoma (which were high) and histologically non-malignant cervices (which were low). Glucuronidase values for endometrial carcinoma, however, were within the range of non-malignant endometrium. Decidua had its greatest activity in early pregnancy; values over 1000 γ per gm. per hour of incubation being obtained from patients under 12 weeks pregnant. It is of interest that the activity in non-malignant and malignant endometrium and decidua are much greater than in any other genital tissue, and some of the highest obtained in the body. Four cases of primary squamous celled carcinoma of the vagina and two of the vulva were studied. In five of these β-glucuronidase activity was increased. A radical vulvectomy was performed on one patient with vulvar carcinoma. The glucuronidase activity of the vulvar lesion and a histologically involved lymph node were high and comparable.

As to the non-malignant cervix histological study revealed a variety of diagnoses. Among these, in addition to histologically normal cervix, were acute and chronic cervicitis with Nabothian cysts, cervical erosion, and leukoplakia. No correlation could be found between the types of benign lesion and the activity of β-glucuronidase in the tissue. Malignant cervical tissues, however, were greater in the proliferative (exophytic) growths than in ulcerative (endophytic) types. As to the non-malignant endometrium histological study revealed all degrees of normal, hypoplastic and hyperplastic growth. Glucuronidase activity was greatest in endometrial hyperplasia. All endometrial tissues were obtained incident to hysterectomy or at the time of diagnostic curettage. The range of β-glucuronidase activity in these specimens was greater (upward) than that reported for endometrium from women with normal menstrual cycles (5).

Serial determinations showed a progressive increase in glucuronidase activity from the portio of the cervix to the endometrium, but little difference between various sites of endometrium (Table 3). Irradiation caused a decrease in tissue β-glucuronidase activity (Table 4). A cervix which was histologically involved with cancer was higher in activity than uninvolved portions. As

[1] β, since glucuronic acid occurs only in the beta position in nature.

[2] Histologic preparations were made from tissues adjoining those assayed. Actively growing, non-necrotic tumor tissue was selected.

such, these data on tissues indicate a significant difference between the glucuronidase activity of non-maligant (non-pregnant) and malignant cervix, no difference between non-malignant and malignant endometrium, and marginal differences in vulva and vagina.

In the light of these findings, one would expect the vaginal fluid, which bathes the cervix and vagina, to become a rich source of this enzyme in the presence of genital malignancy. Using an immunologic pipette 0.1 ml. portions of vaginal fluid were suspended in 3 ml. of distilled water. Assays were made on both uncentrifuged specimens and on supernatants (Fig. 1). In clinically benign conditions, there was a wide distribution of values in uncentrifuged specimens. If 300 γ per ml. vaginal fluid per hour of incubation is used as a practical line of demarcation between a positive and negative test, the per cent of false positives was 18. Uncentrifuged specimens were higher than centrifuged supernatants (Fig. 1), thus localizing some of the activity of the enzyme in the solid component of the suspension. False positive tests (above 300 γ) were found during pregnancy, in menstrual blood, and in vaginitis. The latter observation suggests bacterial action but does not explain the close correlation with tissue activity in cancer patients. Untreated cervical cancer was associated in every case with a high activity (over 320 γ per ml. vaginal fluid per hour) of enzyme in the uncentrifuged vaginal fluid suspension. Following treatment this activity decreased as evidenced by the collective data[3] (Fig. 1) and by the changes observed in individual cases (Table 4). Care must be employed to avoid contamination of the vaginal fluid with lubricating jelly and fresh blood. Likewise, douches previous to examination are discouraged. If added *in vitro* venous blood will inhibit the activity of glucuronidase in vaginal fluid. This inhibition, however, is not always observed when the bleeding originates from the corpus, possibly because of the high activity of glucuronidase in malignant and benign endometrium. Of the two methods, tissue *versus* vaginal fluid assay, the latter (vaginal fluid) is easier and more practical for large surveys.

TABLE 1

β-GLUCURONIDASE ACTIVITY IN UNTREATED GENITAL CANCER

		γ OF β-GLUCURONIDASE ACTIVITY								
		Tissue			Vaginal Fluids†					
					Uncentrifuged			Centrifuged		
DIAGNOSIS	No. of cases	Max.	Min.	Mean*	Max.	Min.	Mean*	Max.	Min.	Mean*
Squamous cell carcinoma of cervix	18	4200	512	1641	1930	320	1020	1300	62	467
Adenocarcinoma of fundus	8	11930	1352	6774	1218	480	835	493	16	227
Squamous carcinoma of vagina	4	1440	680	891	976		976			
Squamous carcinoma of vulva	2	925	237	581						

Figures express γ of phenolphthalein liberated per gm. of tissue or ml. of vaginal fluid per hour of incubation. Only untreated cases are included.

* Arithmetic mean.

† Vaginal fluid was available for assay on 14 cervical and 5 fundal carcinomas, and 1 vaginal carcinoma.

TABLE 2

FREQUENCY TABLE OF β-GLUCURONIDASE ACTIVITY IN BENIGN TISSUES

	Cervix		Endometrium				
γ OF ACTIVITY	Non-pregnant	Pregnant	Non-pregnant	Pregnant	Vulva	Vagina	Ovary
1– 100	16	3				1	1
101– 200	16	1			1	2	1
201– 300	6	5					
301– 400	3	3		1	1	2	2
401– 500		2		1			4
501– 600		2		1			1
601– 700		1		1	1		
701– 800		1			1		1
801– 1000				2			
1001– 3000			3	1			1
2001–10000			31	3			
10000–20000			5	1			
Over 20,000			3				

Figures express γ of phenolphthalein liberated per gm. of tissue per hour of incubation. Figures for additional benign tissues were: Corpus luteum 2218, 958, 1820; dermoid 2530; pseudomucinous cystadenoma 187,338; fibromyomata 0, 3, 82, 402; endometrial polyp 4360, 1990, 1052; fallopian tube 384, 402, 142; myometrium 319, 173, 607, 531.

TABLE 3

SERIAL DETERMINATIONS OF β-GLUCURONIDASE ACTIVITY

Identification	Cervix	Endocervix	Internal os	Endometrium (mid-portion)	Endometrium (fundus)
408607	126	248			10100
81911	48	38	92	4760	3450
435151	118	136	1520	4180	8160
377895	106	178	365	8040	7090
450707	55	95			
*460805	133	143		993	

Figures express γ of phenolphthalein liberated per gm. of tissue per hour of incubation.

* Porro section.

DISCUSSION

It has been suggested that the activity of β-glucuronidase is related to cellular proliferation (14). It seems pertinent that its activity is higher in those normal genital organs (endometrium and corpus luteum) in which mitotic activity is usually present. In addition, the activity of β-glucuronidase has been closely associated with glucuronide formation (2, 3), notably with estrogen (4).

Since the range of activity of β-glucuronidase in malignant tissue is increased over the non-malignant in cervix but not in endometrium, the role of

[3] The collective data included all treated cases of genital cancer (cervix, uterus, and vulva).

this enzyme in cancer tissue becomes a point of interest. If glucuronidase is primarily concerned with the conjugation of estrogen, is endometrial carcinoma different from non-malignant endometrium in its metabolic requirement for steroid? In fact, the outstanding feature of an increased activity of β-glucuronidase in genital cancer, in non-malignant endometrium, in corpus luteum, and in pregnancy (6, 7) has been its close association with cellular growth.

TABLE 4

EFFECT OF IRRADIATION ON β-GLUCURONIDASE ACTIVITY

Identification	Age	Group	Tissue	Vaginal fluid U	Vaginal fluid C	Date	Remarks
460648	56	II	1308			1-20-49	
				1860	1300	2- 5-49	4572 mg. hr.
				540	113	2-18-49	1560 mg. hr.
				275	176	2-27-49	
			398	268	100	3-11-49	
460099	62	III	1537	134	107	1-29-49	Bloody fluid
				808	136	2- 5-49	4560 mg. hr.
						2-21-49	1560 mg. hr.
				900	197	3- 2-49	1560 mg. hr.
			877, 239	329		3-18-49	
462123*	67		1352	480	346	2- 9-49	
				530	365	2-27-49	4560 mg. hr.
			798	231	133	3-14-49	

The table includes irradiation treatment in mg. hours of radium and pertinent notes. Figures express γ of phenolphthalein liberated per gm. of tissue or ml. of vaginal fluid per hour of incubation.

Group—League of Nations Classification; U—Uncentrifuged suspensions of vaginal fluid and water; C—The supernatant fluid after centrifugation.

* Adenocarcinoma of fundus with cervical extension.

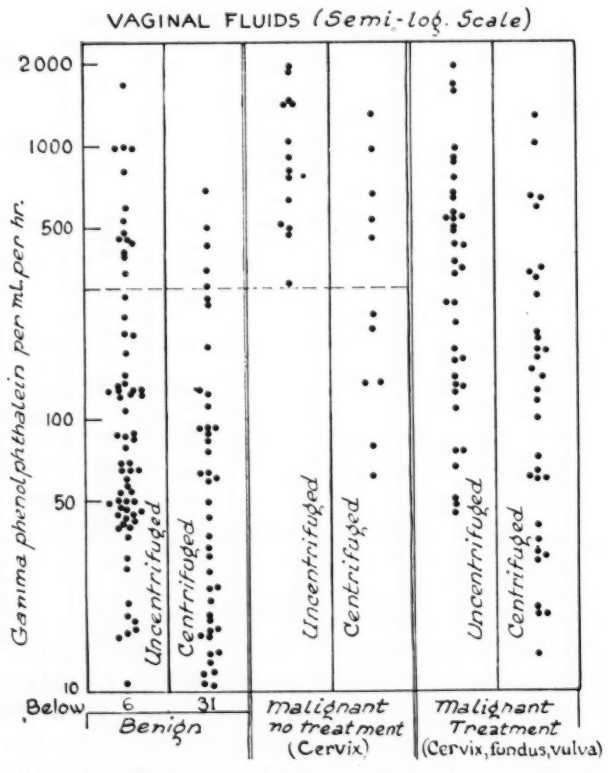


FIG. 1.—Illustrates γ of phenolphthalein liberated per ml. vaginal fluid per hour of incubation.

It would not seem to be likely. If glucuronidase is related to cellular growth, the similarity between non-malignant and malignant endometrium, together with the increased activity in cervical, vaginal and vulvar cancer, is understandable, since all of these are characterized by mitotic figures.

Micro-Kjeldahl determinations were made on homogenized tissues for total protein. Some direct relationship was observed between the total protein of the homogenate and the activity of the enzyme (15). The interpretation of this observation would include the possibility that the activity of β-glucuronidase is related to the cellular structure of the tissue assayed.

SUMMARY

As to tissues, the activity of the enzyme β-glucuronidase is greater in malignant cervix than in non-malignant cervix. In adenocarcinoma of the uterus, however, glucuronidase activity does not differ from that found in endometrium from women with benign uterine bleeding. Increased activity of this enzyme is present in some pregnant cervices.

As to vaginal fluid, assays on the uncentrifuged suspensions showed consistently high values in the presence of untreated cervical cancer. In the presence of clinically benign conditions, false positive tests occurred in 18 per cent of the material studied.

Following irradiation treatment for cervical cancer, the activity of β-glucuronidase in the tissue and in the vaginal fluid declined.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Wm. J. Dieckmann for encouraging this project. The authors also wish to thank Drs. Herbert E. Schmitz and John J. Conley of the Mercy Hospital, Chicago, Illinois for additional material from cancer patients. The senior author (L.D.O.) wishes to thank Dr. Wm. H. Fishman and Miss Rosemary Brunetti for instruction in their method of β-glucuronidase assay.

REFERENCES

1. MARRIAN, G. F. Conjugated Estrogens. Cold Spring Harbor Symposia Quant. Biol., **5**:16–24, 1937.
2. FISHMAN, W. H. Studies on β-Glucuronidase: Increase in β-Glucuronidase Activity of Mammalian Tissues Induced by Feeding Glucuronidogenic Substances. J. Biol. Chem., **136**:229–236, 1940.
3. FLORKIN, M., CRISMER, R., DUCHATEAU-BOSSON, G., and HOUET, R. Glucuronides and β-Glucuronidase. Enzymologia, **10**:220–223, 1942.
4. FISHMAN, W. H. β-Glucuronidase: Its Relation to the Action of Estrogenic Hormones. J. Biol. Chem., **169**:7–15, 1947.
5. ODELL, L. D., and FISHMAN, W. H. Studies on β-Glucuronidase: I. Activity in Human Endometrium. In press.
6. MCDONALD, D. F., and ODELL, L. D. Serum β-Glucuronidase Activity During Normal and Toxemic Pregnancy. J. Clin. Endocrinol., **7**:535–542, 1947.
7. ODELL, L. D., and FISHMAN, W. H. Studies on β-Glucuronidase: III. Normal and Toxemic Pregnancy. In press.
8. FISHMAN, W. H., and ODELL, L. D. Studies on β-Glucuronidase: II. Inhibition of the Decline Postpartum by Stilbestrol. In press.
9. FISHMAN, W. H., and ANLYAN, A. J. Comparison of the β-Glucuronidase Activity of Normal, Tumor and Lymph Node Tissues of Surgical Patients. Science, **106**:66–67, 1947.
10. FISHMAN, W. H., and ANLYAN, A. J. β-Glucuronidase Activity in Human Tissues. Cancer Research, **7**:808–818, 1947.
11. FISHMAN, W. H. Anti-glucuronidase. Variations in Blood and Tissue. Abstracts Chicago Meeting Amer. Chem. Soc. p. 3C, April, 1948.
12. ODELL, L. D., and BURT, J. C. β-Glucuronidase Activity. In press.
13. FISHMAN, W. H., SPRINGER, B., and BRUNETTI, R. Application of an Improved Glucuronidase Assay Method to the Study of Human Blood β-Glucuronidase. J. Biol. Chem., **173**:449–456, 1948.
14. KERR, L. M. H., LEVVY, G. A., and CAMPBELL, J. G. β-Glucuronidase and Tissue Proliferation. Nature, **160**: 572, 1947.
15. BURT, J. C., and ODELL, L. D. In press.

Preparation of a Radioactive Iodotetrazolium Salt and Its Distribution in Mice*†

ARNOLD M. SELIGMAN, M.D., RALPH GOFSTEIN, B.S., AND ALEXANDER M. RUTENBURG, M.D.

(From the Kirstein Laboratory for Surgical Research, Beth Israel Hospital, Boston, and the Department of Surgery, Harvard Medical School, Boston, Massachusetts)

Neoplastic tissues are said to reduce triphenyl tetrazolium chloride to a colored formazan more rapidly and completely than normal tissues (21). This difference was attributed to a higher aerobic glycolytic activity in tumors. If this is indeed the case, it is important to know whether or not tumors reduce tetrazolium compounds *in vivo* more rapidly and extensively than normal tissue. Tetrazolium salts are toxic in mammals (15). Mice will only tolerate the intravenous injection of 0.03 to 0.08 mg. This quantity of a colored pigment (formazan) would not be detectable in the tissues of a mouse. For this reason, a tetrazolium salt labelled in one benzene ring with radioactive iodine (I^{131}) was prepared, and the distribution of radioactivity in the tissues of normal and tumor-bearing mice was determined after intravenous injection.

Diphenyl *p*-iodophenyl tetrazolium chloride (IV) was prepared by a modification of the method for the synthesis of triphenyl tetrazolium chloride (12, 10). Aniline (1 mg.) was iodinated with radioactive iodine and diazotized, in a single reaction vessel (equations 1 to 3) (19). The diazonium compound (I) was coupled with benzal phenylhydrazone (II) (5) in pyridine, to yield a dark red formazan (III). The latter was oxidized with amyl nitrite and hydrochloric acid to the tetrazolium salt (IV).

EXPERIMENTAL

*Preparation of 2,5-diphenyl-3-*p*-radioiodophenyl tetrazolium chloride (IV).*—Iodination of aniline (13) and diazotization of the amine was accomplished with 0.02 millimole of iodine (excess) and 0.01 millimole of aniline according to equations 1 to 3. Reactions 1 and 3 must be conducted in strong acid, and reaction 2 in excess bicarbonate (19).

1) $6NaI + 2NaNO_2 + 8HCl \rightarrow 3I_2 + 8NaCl + 4H_2O + N_2$

2) $I_2 + C_6H_5NH_2 + NaHCO_3 \rightarrow IC_6H_4NH_2 + NaI + H_2O + CO_2$

3) $6IC_6H_4NH_2 + 6NaI + 8NaNO_2 + 20HCl \rightarrow 6IC_6H_4N_2Cl + 3I_2 + 14NaCl + 16H_2O + N_2$

* This investigation was aided by a research grant from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.

† Acknowledgment is due Mr. Myron Milden for technical assistance.

Carrier free I^{131} (3 to 4 mc.) in the form of the sodium salt was added to a solution of 6 mg. sodium iodide in a graduated conical centrifuge tube. The final volume was 2 cc. Free iodine was produced by adding 1.0 per cent sodium nitrite (0.1 cc.) and 10 per cent hydrochloric acid (0.1 cc.). The mixture was shaken and crystalline iodine separated (equation 1). After a few minutes 10 per cent sodium bicarbonate (0.1 cc.) followed by 1 per cent aniline (triply distilled from zinc dust) solution (0.1 cc.) was added. The color of iodine was discharged in the course of one hour as the aniline was iodinated (equation 2). The reaction mixture was cooled to 0° C. and diazotization was accomplished by adding 1 per cent sodium nitrite (0.3 cc.) and 10 per cent hydrochloric acid (0.5 cc.) (equation 3). A chilled solution of 10 mg. benzal phenylhydrazone (I) in 2 cc. pyridine was added to the solution of the diazonium salt (II). A dark red color was produced. The mixture was cooled in ice for 20 minutes and distilled water (10 cc.) was added slowly to precipitate the formazan (III). The mixture was centrifuged for 10 minutes at 2000 r.p.m., the supernatant was discarded, and the red precipitate was washed with three 10 cc. portions of water in order to remove pyridine and inorganic salts. The washed formazan was dissolved in absolute alcohol (3 cc.) and oxidized to the tetrazolium salt (IV) by the addition of amyl nitrite (3 drops) and of concentrated hydrochloric acid (3 drops). The alcohol solution was evaporated in a hot water bath to half its original volume, water (2 cc.) was then added, and the remainder of the alcohol was evaporated. The final volume was approximately 2 cc. The solution was cooled and shaken with chloroform (7 cc.). The aqueous layer was discarded (pipette), and the chloroform extract was concentrated to 3 cc. The tetrazolium chloride (IV) was precipitated by the addition of dry ether. It was washed 3 times with ether (centrifuge) and dried; yield 2.8 to 4.0 mgs. (sinters 160°, decomp. 170°, uncorr.).

A non-radioactive specimen was prepared in the same way on a larger scale. The formazan (III) after recrystallization from dilute alcohol melted at 195°–196° C. (uncorr.). The melting point of the tautomer prepared from *p*-iodophenyl hydrazine (4) is reported to be 185°–186° C. The tetrazolium salt (IV) was soluble in alcohol, chloroform, and water. In aqueous solution, it was reduced to the insoluble formazan with ammonium sulfide.

Analysis calculated for $C_{19}H_{14}N_4ClI$: C, 49.48; H, 3.03;

Found: C, 49.52; H, 3.16

Distribution of Radioactivity in Tissues of Mice.—The distribution of radioactivity in tissue at intervals after intravenous injection of radioactive iodotetrazolium chloride (IV) was determined by methods reported elsewhere (20, 18, 17, 16). One experiment (0.1 mg.) was performed with normal Swiss mice. Two experiments were done with Swiss mice 12 to 14 days after subcutaneous transplantation of sarcoma 37. A sublethal dose of the radioactive iodotetrazolium chloride (IV), which on a molar basis was one-fourth as toxic as triphenyl tetrazolium chloride (15), was given to each mouse. The doses used were 0.1 mg. and 0.2 mg. in each experiment respectively.

The results are shown in Tables 1 and 2 and are expressed as a ratio in per cent of the radioactivity per 0.2 cc. of blood and 200 mg. of wet tissue, to the radioactivity of 0.2 cc. of blood at zero time, assuming the circulating blood volume to be 10 per cent of the body weight (18, 17).

Following intravenous injection (tail vein) in both normal and tumor-bearing mice, the radioactivity disappeared rapidly from the circulating blood. In 30 minutes the level of radioactivity was 3 to 4 per cent and in 8 hours the level was 0.5 per cent of the activity at zero time. The highest concentration of radioactivity was found in kidney, liver and lung, in that order, after injection of 0.1 mg., while the lungs showed the highest concentration after injection of 0.2 mg. All other tissues, including sarcoma 37, were less radioactive.

The radioactivity of lung declined more slowly than that of other tissues in the first 12 hours (Table 1) and this was also the case with liver and spleen after the injection of the larger dose of radioactive iodotetrazolium salt (Table 2). The radioactivity of mesenteric fat showed a rise in the first few hours after injection and declined slowly thereafter. The accumulation and persistence of radioactivity in fat may have been related to the lipoid solubility of the formazan. A similar rise and fall in radioactivity of thyroid was noted and was produced presumably by the release of ionic iodine from the tetrazolium salt or the formazan.

Radioactivity of sarcoma 37 was less than most tissues and disappeared at about the same rate. There was no appreciable difference in the levels of radioactivity in the tissues of normal and tumor-bearing mice (Table 1).

The rate of disappearance of radioactive ionic iodine from blood and tissues of mice, reported previously (18), was much more rapid than was found with the radioactive iodotetrazolium salt described above.

DISCUSSION

Tumor cells have been reported to derive energy largely from aerobic glycolytic reactions which involve fermentation rather than oxidation (22). However, a comparison of the metabolism of tumors of liver and skin *in vitro* with that of the tissue of origin, revealed a higher degree of both aerobic and anaerobic glycolysis in tumors, although a marked decrease in specialized oxidative functions was observed in the tumors (2). Deficiencies in the various oxidative systems of tumors have been reported (1, 11, 9). The cytochrome content of tumors is deficient (3, 6). These deficiencies might lead to accumulation of reducing substances in neoplastic tissue. Triphenyl tetrazolium chloride was reported to be reduced more extensively by tumor than normal tissue *in*

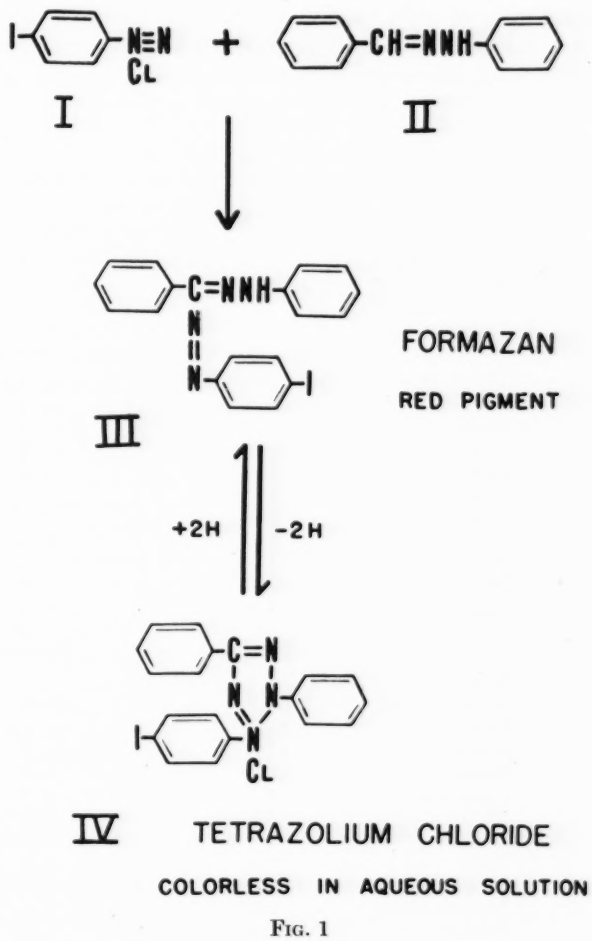


FIG. 1

vitro and by application of the material to the surface of tumor *in vivo* (21). On the other hand, no significant increase in the water-soluble substances capable of reducing methylene blue was found in tumor (14). Ability to decolorize methylene blue was reported to be decreased in neoplastic tissue (1, 7, 8).

In the *in vivo* experiments with radioactive tetrazolium salt, reduction to an insoluble formazan would be expected to result in prolonged localization of radioactivity in the tissues. However, the level of radioactivity was lower in sarcoma 37 than in most normal tissues after intravenous injection. Furthermore, radioactivity disappeared from the tumor at the same rate as with other tissues. The

tissues which normally show high dehydrogenase activity (liver and kidney) were the most radioactive. It may be concluded that sarcoma 37 does not reduce the tetrazolium salt *in vivo* more extensively than most of the normal tissues. The same results were obtained *in vitro* with a homogenate of sarcoma 37 at pH 7.0 to 7.6 (unpublished) (15). Another tetrazolium salt was reduced to its formazan (blue) much more slowly (or not at all) by this tumor than by liver and kidney mash. Although the degree of reduction was increased by anaerobiasis, it was also increased for the normal tissues. Similar results were noted with homogenates of Walker carcinoma 256, of Bagg lymphosarcoma and of several carcinomas from humans (unpublished) (15). No evidence is available from our experiments that neoplastic tissue is able to reduce tetrazolium salts *in vivo* or *in vitro* more readily or extensively than most normal tissues.

SUMMARY

The synthesis of 0.01 millimole of diphenyl *p*-iodophenyl tetrazolium chloride from radioactive iodine (I^{131}) is described. Following the intravenous injection of the radioactive tetrazolium salt into normal and tumor-bearing mice, radio-

TABLE 1

RADIOACTIVITY OF BLOOD (0.2 CC.) AND TISSUES (200 MGS.) OF EACH OF 8 NORMAL AND 8 TUMOR-BEARING MICE, EXPRESSED IN PER CENT OF THE RADIOACTIVITY OF BLOOD AT ZERO TIME MEASURED AT INTERVALS AFTER INTRAVENOUS INJECTION OF 0.1 MG. DIPHENYL *p*-RADIOIODOPHENYL TETRAZOLIUM CHLORIDE

Tissue	Hours 0.5	2	4	8	12	24	48	96
Normal Mice								
Blood	3.4	2.6	1.4	0.5	0.4	0.07	0.08	0.09
Kidney	58.6	34.9	16.8	6.6	4.8	1.2	0.4	0.1
Liver	20.3	18.2	11.4	4.1	3.8	0.7	0.3	0.2
Lungs	12.7	15.2	11.0	5.8	6.4	1.2	0.8	2.1
Spleen	4.2	3.4	2.4	0.8	0.6	0.1	0.1	0.06
Intestine	8.5	6.1	3.5	1.2	1.1	0.1	0.08	0.2
Muscle	3.1	3.7	2.1	1.0	1.4	0.8	0.5	0.5
Nodes	0.4	0.4	0.4	0.1	0.5	0.3	0.1	0.2
Brain	0.1	0.4	0.5	0.4	0.5	0.3	0.1	
Mesenteric fat	0.9	4.8	1.8	2.0	5.1	3.1	2.6	1.9
Thyroid	1.6	3.2	1.4	0.8	12.5	3.1	1.2	3.4
Tumor-bearing Mice								
Blood	3.7	3.3	1.7	0.6	0.4	0.1	0.1	0.1
Kidney	92.2	38.9	22.2	23.2	4.8	1.0	0.6	0.3
Liver	24.5	20.3	15.1	4.1	3.4	1.0	0.5	0.4
Lungs	15.3	14.0	12.0	6.5	7.0	2.4	1.6	0.5
Spleen	4.5	3.1	2.8	1.0	0.08	0.3	0.3	0.2
Intestine	7.8	6.0	5.1	1.4	1.4	0.4	0.4	0.1
Muscle	2.8	3.0	2.4	1.0	1.0	0.5	0.5	0.8
Nodes	0.8	0.4	0.3	0.1	0.3	0.3	0.1	0.3
Brain	0.4	0.4	0.5	0.5	0.5	0.3	0.1	0.1
Mesenteric fat	1.0	7.4	2.1	4.4	3.0	2.8	4.4	0.9
Thyroid	2.0	2.3	2.1	3.1	3.6	2.9	3.2	2.3
Tumor	2.4	1.5	1.9	1.8	1.5	1.6	0.4	0.3

TABLE 2

RADIOACTIVITY OF BLOOD (0.2 CC.) AND TISSUES (200 MGS.) OF EACH OF 8 TUMOR-BEARING MICE EXPRESSED IN PER CENT OF THE RADIOACTIVITY OF BLOOD AT ZERO TIME, MEASURED AT INTERVALS AFTER INJECTION OF 0.2 MG. DIPHENYL *p*-RADIOIODOPHENYL TETRAZOLIUM CHLORIDE

Tissue	Hours 0.5	2	4	8	16.5	24	49	120
Blood	4.5	2.9	2.3	0.6*	0	0	0	0
Kidney	45.3	34.4	26.9	13.2	6.4	3.9	2.7	0.8*
Liver	34.6	26.6	24.8	15.0	10.3	9.0	9.4	7.6
Lungs	111.5	84.4	55.6	48.2	46.4	20.9	30.2	22.2
Spleen	8.3	8.1	6.1	2.8	4.4	4.8	3.9	2.5
Intestine	6.5	3.2	9.7	2.0	0.5*	0.5*	0	0
Muscle	2.5	1.4*	1.1*	1.3*	0.4*	0.5*	0	0
Mesenteric fat	1.2*	1.4*	3.4	5.2	0.9*	2.0	1.7	1.3*
Thyroid	3.1	12.3	6.5	5.7	4.3	9.5	4.0	5.0
Tumor	2.4	1.6	2.3	1.8*	0.9*	0	0	0

* Less than 3× background.

activity disappeared rapidly from the circulating blood, and appeared in greatest amount in kidney, liver, and lung. Sarcoma 37 contained less radioactivity than most other tissues.

REFERENCES

1. CHALKLEY, H., and GREENSTEIN, J. P. Effect of Nucleates on Dehydrogenase Systems. J. Nat. Cancer Inst., **6**:119–141, 1945.
2. DICKENS, F., and WEIL-MALHERBE, H. The Metabolism of Normal and Tumor Tissue. XX. A Comparison of the Metabolism of Tumors of Liver and Skin with That of Tissue of Origin. Cancer Research, **3**:73–87, 1943.
3. DUBOIS, K. P., and POTTER, V. R. Biocatalysts in Cancer Tissue. I. Cytochrome C. Cancer Research, **2**:290–293, 1942.
4. FICHTER, FR. and Philipp K. Studien in ungesättigten Säuren. IV. Über jodierte Phenylhydrazine. J. f. prakt Chemie (N.F.), **74**:312–316. 1906.
5. FISCHER, E. Ueber aromatische Hydrazinverbindungen Ber. d. deutsch. chem. Gesellsch., **9**:880–891, 1876.
6. GREENSTEIN, J. P. Biochemistry of Cancer. New York: Academic Press, Inc. 1947.
7. GREENSTEIN, J. P., and CHALKLEY, H. W. Note on Some Aspects of the Effects of Nucleates in Primary and Transplanted Rat Hepatomas. J. Nat. Cancer Inst., **6**:207–209, 1946.
8. HEINLEIN, H. Die Oxydoreduction maligner Tumoren. Ztschr. f. Krebsforsch., **30**:506–512, 1930.
9. KIDD, J. G., WINZLER, R. J., and BURK, D. Comparative Glycolytic and Respiratory Metabolism of Homologous Normal, Benign, and Malignant Rabbit Tissues with Particular Reference to the Benign Virus Papilloma (Shope) and a Transplanted Cancer Derived Therefrom (the V_2 Carcinoma). Cancer Research, **4**:547–553, 1944.
10. KUHN, R., and JERCHEL, D. Über Invertseifen VII Mitteil: Tetrazolium Salze. Ber. d. deutsch. chem. Gesellsch., **74**:941–948, 1941.
11. MAYER, N. Studies in Cancer. X. Oxidative Capacity of Tumors. Cancer Research, **4**:345–348, 1944.
12. PECHMAN, H. V., and RUNGE, P. Oxydation der Formazylverbindungen. Ber. d. deutsch. chem. Gesellsch., **27**: 2920–2930, 1894.
13. *p*-iodoaniline: Organic Syntheses. Collective Vol. II, p. 347. New York: John Wiley & Sons, Inc.; London: Chapman and Hall Limited, 1943.
14. RIEHL, M. A., and LENTA, M. P. Dehydrogenase Studies of Tissues from Normal and Tumor Bearing Mice. I. Total Dehydrogenase Activity. Cancer Research, **9**:42–46,1949.
15. RUTENBURG, A. M., GOFSTEIN, R., and SELIGMAN, A. M. Preparation of a New Tetrazolium Salt Which Yields a Blue Pigment on Reduction and Its Application to the Demonstration of Enzymes in Normal and Neoplastic Tissues. In preparation.
16. RUTENBURG, A. M., SELIGMAN, A. M., and FINE, J. Studies with Radioactive Iodized Fat. I. Preparation of Radioactive Fat with Observations on the Absorption of Fat Following Subcutaneous and Intraperitoneal Injection in Dogs. In preparation.
17. RUTENBURG, A. M., SELIGMAN, A. M., and FINE, J. Studies with Radioactive Iodized Fat. II. The Tissue Distribution of Emulsified Fat Following Intravenous Administration. In preparation.
18. SELIGMAN, A. M., RUTENBURG, A. M., and FRIEDMAN, O. M. Distribution Studies in Mice Following the Intravenous Injection of Diethyl β-iodoethyl Amine Hydrochloride Prepared with Radioactive Iodine. J. Nat. Cancer Inst., 9: 261–270, 1949.
19. SELIGMAN, A. M., and SACK, T. Chemical Alteration of Polysaccharide from *Serratia marcescens*. III. Reaction of Polysaccharide with Radioactive *para* Iodobenzene Diazonium Chloride and the Use of the Product in an Experimental and Clinical Study. In preparation.
20. SELIGMAN, A. M., SHEAR, M. J., LEITER, J., and SWEET, B. Chemical Alteration of Polysaccharide from *Serratia marcescens*. I. Tumor Necrotizing Polysaccharide Tagged with Radioactive Iodine. J. Nat. Cancer Inst., **2**:13–18, 1948.
21. STRAUS, F. G., CHERONIS, N. D., and STRAUS, E. Demonstration of Reducing Enzyme Systems in Neoplasms and Living Mammalian Tissues by Triphenyl Tetrazolium Chloride. Science, **108**:113–115, 1948.
22. WARBURG, O. Metabolism of Tumors. Translated by F. Dickens, p. 327. London: Constable & Co., 1930.

A Statistical Study of Tumors Among Koreans

I. S. YUN, M.D.

(From the Graduate School, Seoul National University, Seoul, Korea)

Few statistical studies have been made on incidence and types of tumor in Koreans. Choy (1) in 740 microscopic diagnoses found that the female breast was the commonest site of primary tumor and that cancer of the penis was second. Ludlow (2) reported that carcinoma of the stomach was as common in the Korean as in other peoples, that the incidence of carcinoma of the uterus was about the same as in other countries, and that carcinoma of the penis was unusually common. These tumors were first, second, and third, respectively. Similar figures are reported from China and Siam.

The present report is a study of tumors seen during the fifteen years from 1925 to 1939. The materials were obtained mostly from our hospital, but some were collected from various mission hospitals from all over the country. Most of them were surgical specimens, but a small number of necropsies is included.

Our total pathological accessions number 3254. These include 409 (12.5 per cent) benign tumors and 632 (19.4 per cent) malignant tumors.

BENIGN TUMORS

The total number of benign tumors was 409, of which 71 were found in males and 338 in females, a sex ratio of 1:5. The favorite sites were: ovaries, 116 (28.5 per cent); uterus, 103 (25.5 per cent); breast, 49 (11.2 per cent); thyroid, 32 (7.8 per cent). These tumors were classified histologically as follows: cyst, 116 (28.5 per cent); fibroma, 98 (24.0 per cent); papilloma, 32 (18.5 per cent); polyp, 30 (7.3 per cent).

The 116 ovarian cysts occurred at all ages from 18 to 77 years. The average age was 39.6 years. The greatest number was seen in the age period 41 to 45 years, followed by ages 26 to 30 and 36 to 40 in that order.

The 98 fibromas were found in many locations and at all ages, the youngest occurring in the thigh of a 3-year-old boy. The oldest was in the uterus of a female 68 years old. The average age was 42.4 and the ratio of males to females was 1:3.5. The commonest site was the uterus (45 cases), followed by breast and then by ovaries.

Thirty-two benign tumors were found in the thyroid gland. Of these, 7 occurred in males and 25 in females. The ages varied from 19 to 55 years.

Papillomas from various sites numbered 32 cases, of which 15 were in males and 17 in females. The youngest was a 5-year-old male (nasal cavity). The oldest was a 64-year-old male (bulbar cavity). The average age was 34.8 years. The commonest site was the rectum (18.7 per cent).

One hundred and three benign tumors were found in the uterus. They were classified histologically as follows: fibroma, 45 cases (uterine body, 31; uterine cervix, 14); fibromyoma, 25 cases (uterine body, 22; uterine cervix, 3); adenomyoma, 14 cases (all uterine cervix); myoma, 13 cases (uterine body, 9; uterine cervix, 4); papilloma, 5 cases (uterine body, 1; uterine cervix 4); angioma, 1 case (uterine cervix).

MALIGNANT TUMORS

There were 632 malignant tumors, of which 429 (67.8 per cent) were carcinomas, 183 (28.8 per cent) were sarcomas, and the other 20 (13.4 per cent) were miscellaneous malignant tumors.

1. *Carcinoma.*—One hundred and eighty-nine carcinomas occurred in males and 240 in females. There was a gradual rise in the number of cases to a maximum in the decade 51 to 60 years (36.6 per cent) and an abrupt decrease thereafter. The topographical distribution of these carcinomas was as follows: uterus, 81 cases (18.8 per cent); breast, 70 cases (16.3 per cent); stomach, 56 cases (13.0 per cent); skin, 53 cases (12.3 per cent); penis, 36 cases (8.3 per cent); tongue, 24 cases (5.5 per cent); liver, 24 cases (5.5 per cent). The favorite sites in the male were penis, stomach, skin, liver, and tongue in the order given. In the female the commonest sites were uterus, breast, stomach, skin, and vulva in that order.

Carcinoma of the uterus was represented by 81 cases, which was 18.8 per cent of all carcinomas. The ages were given from 22 to 81 years. The greatest number of cases was in the decade 41 to 50, with a decrease thereafter. The ratio between carcinoma of the uterine body and of the uterine cervix was 25:56, or approximately 1:2. The aver-

age age for carcinoma of the uterine body was 52.3 and for the uterine cervix 48.4 years.

The total number of breast cancers was 70 cases, which was 16.3 per cent of all carcinomas. Sixty-nine occurred in females and 1 in a male. The ages varied from 28 to 78, with an average age of 51.1 years. Most cases occurred in the decade of ages 51 to 60. The carcinoma in a male breast was found in a patient 49 years old.

The series included 56 cases of carcinoma of the stomach, which was 13.0 per cent of all carcinomas. Thirty-five cases were in males and 21 in females. The youngest patient was a 24-year-old man with a medullary carcinoma, and the oldest was a man of 76 with a glandular carcinoma. The histological subclassification was as follows: scirrhous carcinoma, 37.5 per cent; adenocarcinoma, 28.6 per cent; medullary carcinoma, 19.6 per cent; gelatinous carcinoma, 14.3 per cent.

There were 53 cases of carcinoma of the skin. This comprised 12.3 per cent of all carcinomas. Thirty-four cases occurred in males and 19 in females. The ages were from 25 to 72, with an average of 52.8 years.

Carcinoma of the penis with 36 cases was 8.3 per cent of all carcinomas. The ages were from 31 to 67 years, with an average of 47.3 years.

There were 24 cases of carcinoma of the tongue. This comprised 5.5 per cent of all carcinomas. There were 19 cases in males and 5 in females. The ages were from 30 to 64, with an average of 47.3 years.

The total number of liver cancers was 24 cases, or 5.5 per cent of all carcinomas. Nineteen occurred in males and 5 in females. The ages varied from 30 to 64, with an average age of 50.4 years.

2. *Sarcoma.*—The total number of sarcomas was 183 cases. This was 19.2 per cent of all malignant tumors. One hundred and nine cases were in males and 74 in females. The incidence curve shows peaks in the decades 11 to 20 and 41 to 50. The topographical locations were as follows: lymph glands, 23.9 per cent; lower limbs, 14.4 per cent; ovaries, 8.9 per cent. The histological subclassification showed round-cell sarcoma in 24.0 per cent; spindle-cell sarcoma, 16.4 per cent; and fibrosarcoma, 15.4 per cent.

3. *Other malignant tumors.*—This group totaled 20 cases, of which 3 were hypernephromas, 11 endotheliomas, 3 chorionepitheliomas, and 3 peritheliomas.

SUMMARY

From the above statistical studies it seems conclusive that tumors among Korean people show similar figures to those in Western countries, with two remarkable exceptions, namely, carcinoma of the liver and of the penis, which show much higher percentages among Koreans.

REFERENCES

1. CHOY, P. D. The Result of 740 Sections Diagnosed. J. Severance Union Medical College, **1**:45–56, 1933.
2. LUDLOW, A. I. Carcinoma in the Korean. China M. J., **43**:465–472, 1929.

Spontaneous Basophilic Tumors of the Pituitary Glands in Gonadectomized Mice*†

MARGARET M. DICKIE, M.A., AND GEORGE W. WOOLLEY, PH.D.

(Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine)

The presence of spontaneous pituitary tumors and lesions following the appearance of adrenal cortical carcinomas presents a new line of evidence for the close relationship of adrenal cortex and pituitary disfunctions in experimental animals and of these disfunctions to tumor formation and their possible inherited tendencies. In man, there are not sufficient data to say conclusively whether the adrenal cortex or anterior pituitary abnormalities are the primary causal agents in the development of various syndromes, such as Cushing's syndrome, (Cushing's disease) and adrenal virilism. In fact the clinical evidence of these syndromes is so confusing that it is difficult to try to ascribe the causal agent to any one endocrine gland and/or its secretions. Kepler (22) recently has given a critical review of Cushing's syndrome and its association with Crooke's changes of the basophile cells of the anterior pituitary, *i.e.* hyalinization of the basophile cells. He made several postulates about the mechanism that might be involved, to be discussed later, but did not definitely say that either the anterior pituitary or the adrenal cortex *was* the primary cause. Hyperadrenocorticism, however, usually plays an essential part in this syndrome. In the experimental animals under discussion in the present report it is *definite* that the adrenal cortical carcinomas precede the gross pituitary abnormalities by several months. The material may be useful in helping to elucidate the intimate interplay of pituitary and adrenocortical hormones.

A review of the literature indicates that spontaneous hypophyseal abnormalities in mice are extremely rare. Slye found only one abnormality in 11,000 mice examined (32). Tumors of the mouse hypophysis interpreted as chromophobe adenomas have been produced following estrogenic treatment (16). Burrows described such a tumor in a male mouse following estrogenic treatment (5). Gardner and Strong have shown that the pituitary response to estrogen treatment was strain limited and that the strain C57 Black mice were more susceptible than other strains (16). Pituitary tumors of the above type were observed following implantation of pellets containing an estrogenic substance, diethylstilbestrol, in strain ce mice in this laboratory (45).

Castration changes in the pituitaries of rats have been described by many workers, Addison (1), Biggart (2), Guyer (20), and Severinghaus (30). They have noted that there is an increase in the number of basophiles and that these basophiles become vacuolated to such an extent that they are called "signet ring" cells (30). These changes, however, have not been found to any extent in the mouse hypophysis following gonadectomy (13).

The effects of early gonadectomy in mice of various strains have been intensively studied by Woolley, Fekete, and Little (14, 41, 42, 43). It was observed that the strains used reacted differently to gonadectomy. Experiments were carried out with several inbred strains, namely, JAX, C57 Black, C57 Brown, dba, ce, A and C3H. Their results will be summarized briefly here. In strains C57 Black, C57 Brown and A the accessory reproductive organs remained small, the submaxillary gland unstimulated, that is, small and dark; no obvious changes in the hypophyses or extensive changes in the adrenals were noted (46). However, the dba and ce mice reacted quite differently. The dba gonadectomized animals developed nodular hyperplasia of the adrenal cortex; this modified cortex seemed to be supplying hormonal stimulation to the organs ordinarily influenced by the hormones of the gonads themselves. The accessory reproductive organs in gonadectomized female mice were stimulated and, as histologically determined, they were feminized (14). A few mammary tumors also appeared in this experimental group. The pituitaries were examined and some abnormalities noted but changes were not studied in detail at that time. The ce mice not only developed nodular hyperplasia of the adrenal cortex but, later, adrenal cortical carcinomas, the latter first appearing in the hyperplastic areas. These carcinomas produced both masculinizing and feminizing hormones as evidenced by the histological picture of the submaxillary glands and the accessory reproductive organs in both gonadectomized sexes (42, 43, 12). Changes

* This work has been aided by grants to the Roscoe B. Jackson Memorial Laboratory by the Commonwealth Fund, the Anna Fuller Fund, the Jane Coffin Childs Memorial Fund for Medical Research, and the National Advisory Cancer Council.

† Animal tissues were collected previous to the fire of October 23, 1947 and most of the tissues were saved. Some detailed notes and slides have been missing since that date.

in the hypophyses were observed in a few mice but not studied intensively.

After differences in the reactions of the various strains to early gonadectomy became known, it was decided to study reactions after gonadectomy on animals of the F_1 generation of reciprocal crosses of some of these strains. As the animals were examined it became evident that frequent hypophyseal abnormalities were occurring several months after the appearance of adrenal tumors in some of the crosses. Concurrent with this, there was very extensive and unusual mammary gland development.

The other data from these crosses will be reported separately and only the pituitary abnormalities and the relationships of the mammary glands to these abnormalities will be discussed in the present report.

MATERIALS AND METHODS

Approximately 800 F_1 mice from reciprocal crosses between dba and C57 Black, dba and ce, ce and C57 Black, and A and C3H strains were gonadectomized from 1 to 3 days after birth. The mice were then returned to the nest and allowed to mature and age without any further treatment. Virgin females and unmated males from these crosses were used for control animals. All animals, experimental and control, were maintained on a diet of Purina fox chow and water *ad libitum*, and at a temperature of approximately 70° F.

Animals from both the experimental and control groups were autopsied at monthly intervals from 15 days and 1 month up to 24 months of age and beyond. Gross and histologic observations were made on the endocrine glands and accessory reproductive organs for comparative studies. The present report is on the macroscopic and histological findings of the pituitary and mammary glands from these animals. Numerous abnormalities were encountered in the hypophyses of some of the experimental animals but not in the control animals. The pituitaries of the control animals and of the experimental animals that did not show gross abnormalities were fixed in 10 per cent formalin in normal saline, embedded in paraffin, and sectioned at 8 micra. Several longitudinal sections were cut from each pituitary and stained with Mayer's haematoxylin and aqueous eosin.

For the study of the abnormal pituitaries a good differential staining method was necessary. Trials with a number of fixatives in conjunction with various staining methods showed that the mouse pituitary was difficult to stain differentially, as compared to the rat or the hamster. A method was finally developed using either a modified Zenker-formol solution or a modified Bouin solution for fixation. The sections were stained with a combination of Mallory's and McFarlane's triple staining methods (11). Abnormal glands were cut in cross section and serially sectioned at 4 micra.

A few normal pituitaries beyond 12 months of age were fixed and stained in the same way as the abnormal pituitaries for purposes of comparison.

The mammary glands were left on the skins of the animals and were fixed in modified Tellyesniczky's fluid. These glands were then studied with the aid of the dissecting microscope and some representative glands from various animals were removed and sectioned. Most of these tissues were stained with haematoxylin and eosin.

GROSS OBSERVATIONS ON THE PITUITARIES

All gross observations on the pituitaries and mammary glands were made with the aid of a dissecting microscope (7 × ocular and 10 × objective).

The mouse hypophysis lies on the sphenoid bone and is covered by a tough membrane, the intrasellar dura (Fig. 1). At autopsy the anterior lobe is usually light pink in color and the intermediate and posterior lobes have an opaque color, the pars

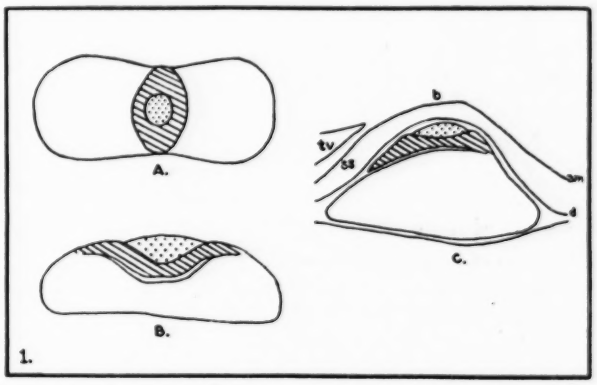


FIG. 1.—Diagrammatic sketches of the mouse pituitary. A. dorsal view of whole normal gland as seen at autopsy. Pars intermedia is lined and pars nervosa is stippled. B. longitudinal section of the gland, the residual lumen is between the pars anterior and the pars intermedia. C. cross section of gland, *tv* the third ventricle, *d* the dura, *ss* the subdural space, *am* the arachnoid membrane, and *b* the brain.

nervosa being slightly whiter than the pars intermedia. Normal hypophyses in mice measure about 2.16 mm. laterally × 1.00 mm. anterioposteriorly. In older mice the glands are slightly smaller, 1.90 mm. × 0.9 mm. (measurement on microscopic sections).

In some of the gonadectomized F_1 mice, the pituitaries were enlarged and nodular. For instance one abnormal gland measured 3.7 × 2.6 mm. Two kinds of nodular areas were noted in these glands. The first type was a well defined opaque nodule or lesion at the periphery of the anterior lobe that did not alter the normal contour of the gland (Fig. 2). The second type was a protruding hemorrhagic nodule (Fig. 3). These nodules could be found at either or both tips of the anterior lobe and sometimes were so extensive that they pushed the intermediate and posterior lobes aside but did not seem to invade them. Characteristically they gave the impression of penetrating deeply into the anterior lobe.

These nodular areas occurred after 14 months of

age, sometimes both kinds in one gland, sometimes only one. Table 1 shows the data on the eight F_1 crosses that were made. It shows that there are strain variations and emphasizes the fact that the pituitary tumors occur at 14 months of age or later. In all cases the individuals that showed hypophyseal tumors had well developed adrenal tumors. The table does not show whether each adrenal possessed a tumor, but includes all the animals that had one or more adrenal tumors.

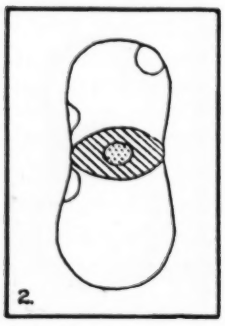


FIG. 2.—Diagram of the mouse pituitary showing possible sites of the opaque nodules.

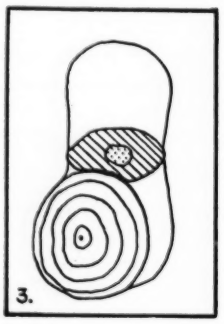


FIG. 3.—Diagram of the mouse pituitary showing a large hemorrhagic nodule that has pushed the pars intermedia aside slightly.

GROSS OBSERVATIONS ON THE MAMMARY GLANDS

Concurrent with the gross hypophyseal changes, there was very extensive alveolar development of the mammary glands. It has been reported previously that following the appearance of adrenal cortical carcinomas in gonadectomized mice of the ce strain there was some mammary development, such as growth and extension of ducts, the appearance of end buds (42, 43) and some alveolar development. Where this latter occurred, it was correlated with abnormal hypophyseal changes (46). The alveolar development that was found along with the hypophyseal changes was very pronounced; the alveoli were large and sometimes the ducts were concealed by the alveolar growth. This growth was similar in gonadectomized animals, both females and males. The only difference was that all five pairs of mammae were present in the gonadectomized females, while in the castrated males some of the glands were absent, probably because the rudiments for some of the glands were never formed.

MICROSCOPIC OBSERVATIONS ON THE PITUITARIES

The Opaque Nodules.—The opaque nodules that occurred in the hypophyses of the experimental animals were not encapsulated. They were composed of densely arranged cells.

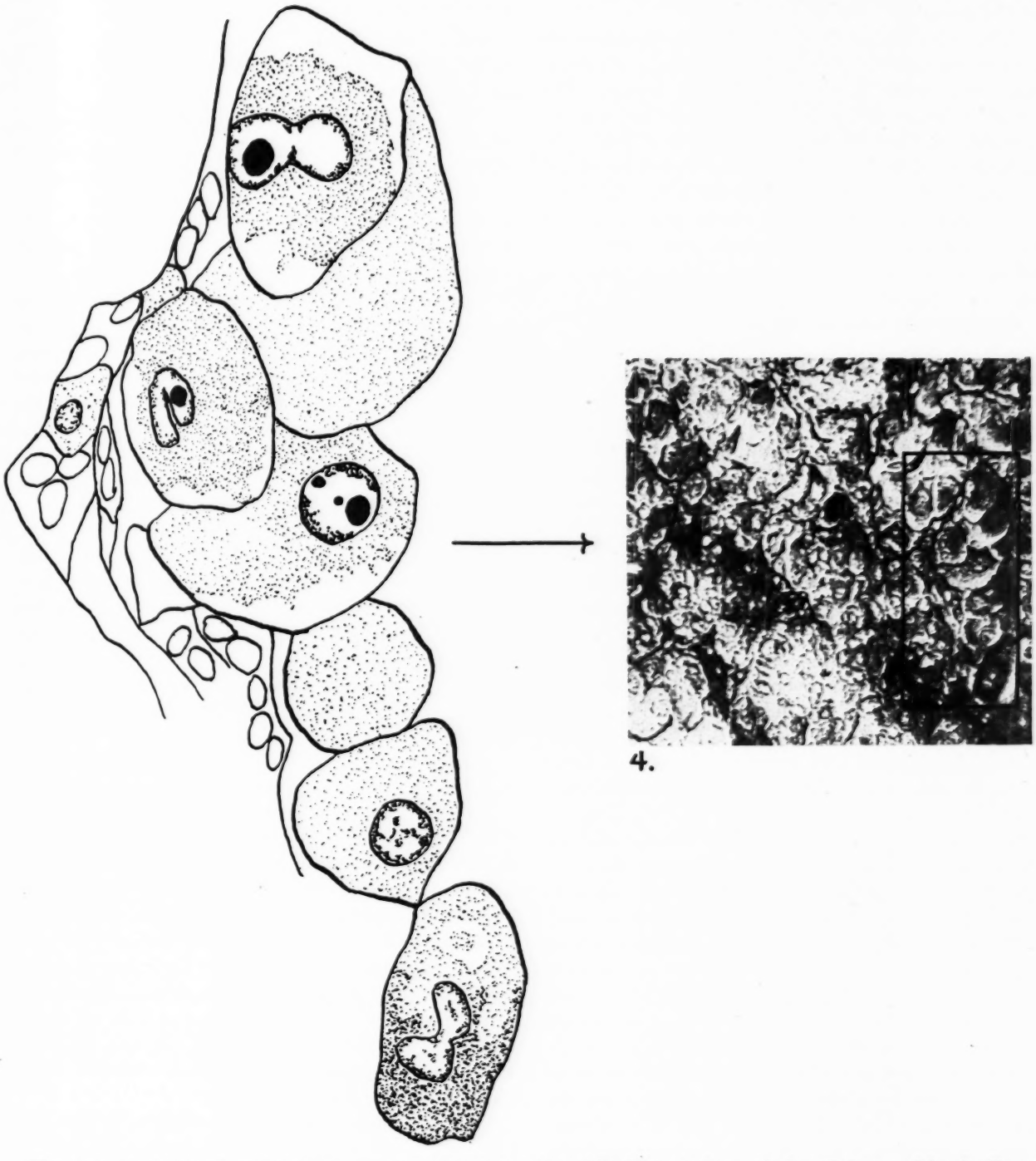
The cells were mainly of two types: (a) small cells, closely packed in disorderly arrangement and, (b) very large cells arranged in small acinar clusters separated from one another by delicate reticular fibers. Both types of cells could be found in one opaque nodule, or there might be only one type in an opaque nodule.

The small cells had light staining cytoplasmic basophilic granules and remnants of a Golgi apparatus comparable in form to the negative image of the Golgi apparatus in the normal mature basophile (*i.e.*, a doughnut shaped negative image associated with basophiles). The nuclei of these cells were slightly different from those found in normal anterior lobe cells. The chromatin was found only in a light ring at the periphery and there was a large yellow-staining nucleolus as well as several very small fuchsinophile nucleoli. While a few fuchsinophile nucleoli are present in normal nuclei, no yellow nucleoli were observed in the nuclei of the cells of normal glands. Some of these

TABLE 1

FINDINGS FOR GONADECTOMIZED MALES AND FEMALES OF EIGHT F_1 CROSSES AT TWO AGE GROUPS

		6 TO 13 MONTHS			14 MONTHS AND BEYOND	
CROSS	SEX	NO. OF MICE	Adrenal tumors	Pituitary tumors	NO. OF MICE	Adrenal tumors	Pituitary tumors
dba ♀ × C57 ♂	♀ ♀	8	0	0	11	9	1
	♂ ♂	8	0	0	16	4	0
C57 ♀ × dba ♂	♀ ♀	8	0	0	16	10	4
	♂ ♂	8	1	0	14	3	0
dba ♀ × ce ♂	♀ ♀	8	5	0	19	19	9
	♂ ♂	8	1	0	24	24	18
ce ♀ × dba ♂	♀ ♀	8	6	0	21	21	12
	♂ ♂	8	1	0	16	16	14
ce ♀ × C57 ♂	♀ ♀	8	3	0	15	14	4
	♂ ♂	8	0	0	15	9	0
C57 ♀ × ce ♂	♀ ♀	8	4	0	20	20	7
	♂ ♂	8	2	0	13	11	2
A ♀ × C3H ♂	♀ ♀	4	0	0	18	14	4
	♂ ♂	4	0	0	13	8	2
C3H ♀ × A ♂	♀ ♀	4	2	0	10	10	1
	♂ ♂	4	0	0	11	11	1



The scale in which all cell sketches are reproduced is 2 cm. = 0.01 mm.

FIG. 4.—Large transitional basophiles and a degranulating basophile. There is degranulation of the transitional cells and several nuclei are lobular. From 18 months old gonadectomized male (ce ♀ × dba ♂) F_1 hybrid WK 450. Mag. ×439.

cells were partially degranulated, only a little cytoplasm being found adjacent to the nucleus and at the periphery of the cell.

The cytoplasmic elements of the large cells were always basophilic and the cytoplasmic elements appeared either finely granular or foamy. The negative image of the Golgi apparatus that was observable by the staining methods used was comparable to that found in normal ripe basophiles, doughnut shaped. Some of these cells had large polymorphic and/or lobular nuclei and some cells were multinucleate. The nucleoli were very large, filling almost the entire nucleus. They stained pale blue and appeared to be vesicular.

Occasionally among the above described small and large cells, very small cells were found with a clear bluish gray cytoplasm and little or no granulation. In the nuclei of the cells, the nucleoli were large and fuchsinophile staining.

There were many variations in the amount of granulation of the cells that comprised the opaque nodules. There were no mitotic figures observed in any of these cells. These cells have been designated transitional basophiles and the opaque nodules shall be called focal basophile adenomas.

The acidophiles were absent in the opaque areas while those that were found adjacent to the opaque nodules were very densely arranged and were somewhat larger than average acidophiles in normal glands. Normal acidophiles ranged from 9.22 micra to 10.41 micra while the acidophiles adjacent to the nodules were from 10.17 micra to 13.81 micra in diameter. Other than the size difference the acidophiles seemed normal. The staining reactions of the α granules seemed normal and no abnormal nuclei were observed. The negative image of the Golgi apparatus which forms a cap over the nucleus in acidophiles also appeared normal.

The Hemorrhagic Nodules.—The nodules, which appeared to be hemorrhagic grossly, were also noncapsulated. They frequently contained hemorrhagic cysts and were surrounded by scattered clusters and strands of abnormal basophilic cells. In some nodules free blood cells were found amid strands of abnormal basophiles. In some areas the basophiles showed evidence of loss of cohesion. These basophiles were coarsely granular and the negative image of the Golgi apparatus was hypertrophied, and filled almost the entire cytoplasmic area. In glands with large hemorrhagic areas and basophiles, usually no other cell types were present.

Other Abnormalities.—There were several other abnormalities that are worthy of mention and that do not fall into the types given above.

Two types of colloid cysts were noted in the hypophyses examined, both occurring in the anterior lobe. The first and most common type was lined by thin epithelial cells. The colloid was usually basophilic, only occasionally being acidophilic. These cysts were usually very small and occurred in clusters or singly.

The second type of colloid cyst was lined by cuboidal ciliated cells (Fig. 9). Some flat nonciliated cells were found occasionally. The appearance of these non-ciliated cells might be due to a gradual transformation from non-ciliated to ciliated cell type. The colloid in these cysts was always basophilic.

From the study of the glands it is evident that cysts are common in the anterior lobe of the pituitary. In the present series they were found most often in control males and gonadectomized females as is shown in Table 2. These are by no means absolute figures since none of the glands of the control animals and only some of the glands of the experimental animals were serially sectioned, therefore many cysts may have been missed.

Another abnormality was found in several pituitaries of gonadectomized and control animals. Groups of two to six cells were surrounded by normal anterior lobe cells. These encircled cells had very large nuclei and the cytoplasm was either very light staining or seemed to be absent. It was thought that they might be degenerating basophiles, but later they were diagnosed as phagocytes.[1]

In a castrate male F_1 (dba ♀ × C57 Black ♂), 21 months of age six giant cells were found.[2]

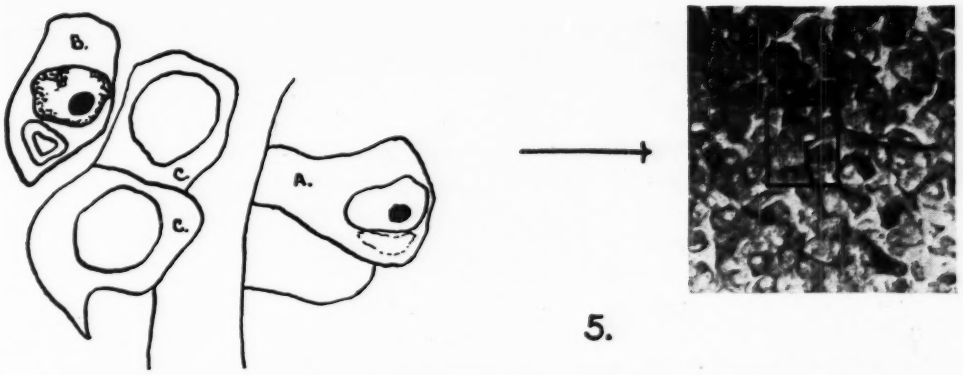
In WK 706, a 26 months old castrate male F_1 (ce ♀ × dba ♂) the pituitary contained a yellow nodule. Microscopic analysis showed that this nodule was composed of cells containing yellow lipochrome pigment. Close to this area cholesterol clefts separated by flattened epithelial cells were found. This area of tissue joined both lobes but did not invade either lobe to any extent. Such cholesteatomas have been described and do occur occasionally in the hypophysis[1, 3]. In the adjacent anterior lobe amyloid degeneration occurred in the blood vessel walls.

The only abnormality that occurred in the control series, other than the hyaline cysts, was a chromophobe adenoma that was found in a 30

[1] Diagnoses were made by Miss Elizabeth Fekete of this laboratory.

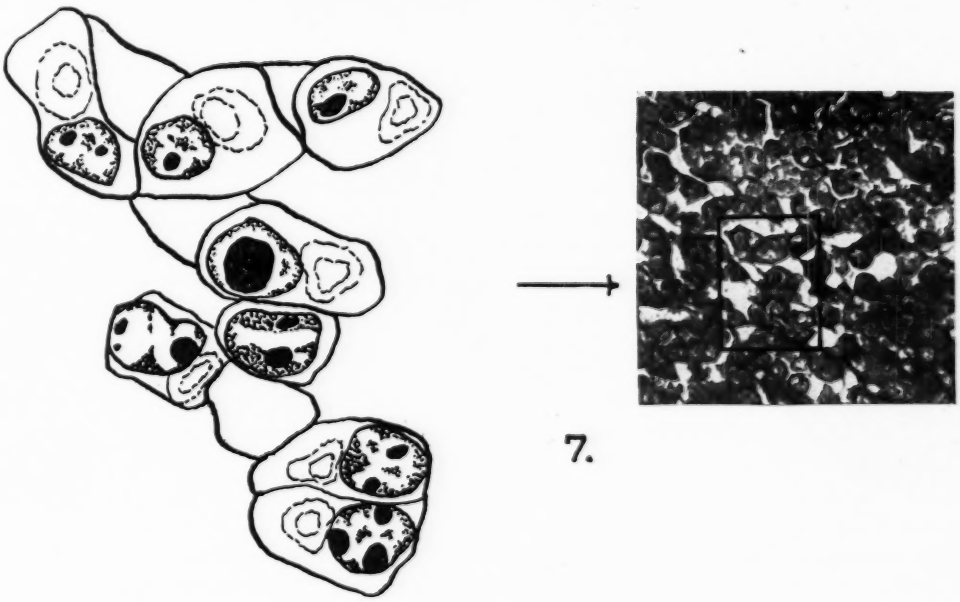
[2] There seems to be no simple explanation as to why these should have been present in the pituitary. Diagnoses by Miss Elizabeth Fekete of this Laboratory.

[3] Cholesteatomas are formed by epithelial cells that were left behind at the time of the closure of the neural crest and grow slowly throughout life to form these benign tumors (10).



5.

6.

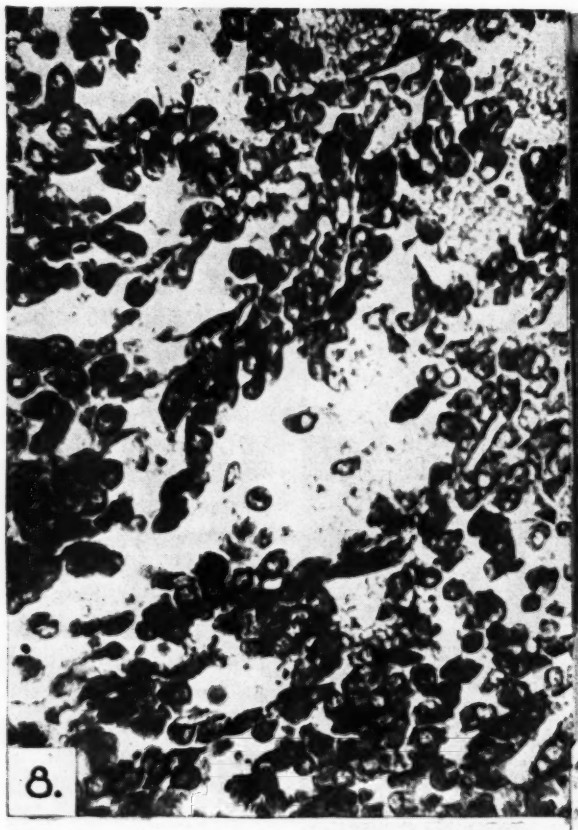


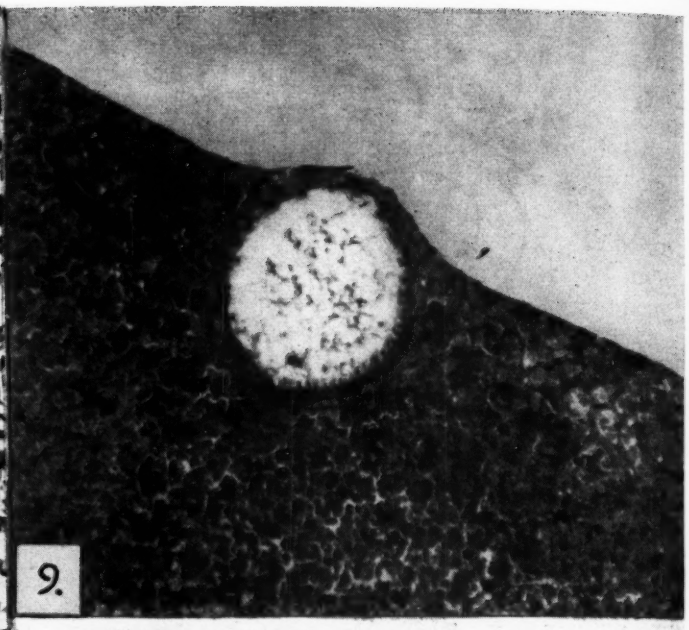
7.

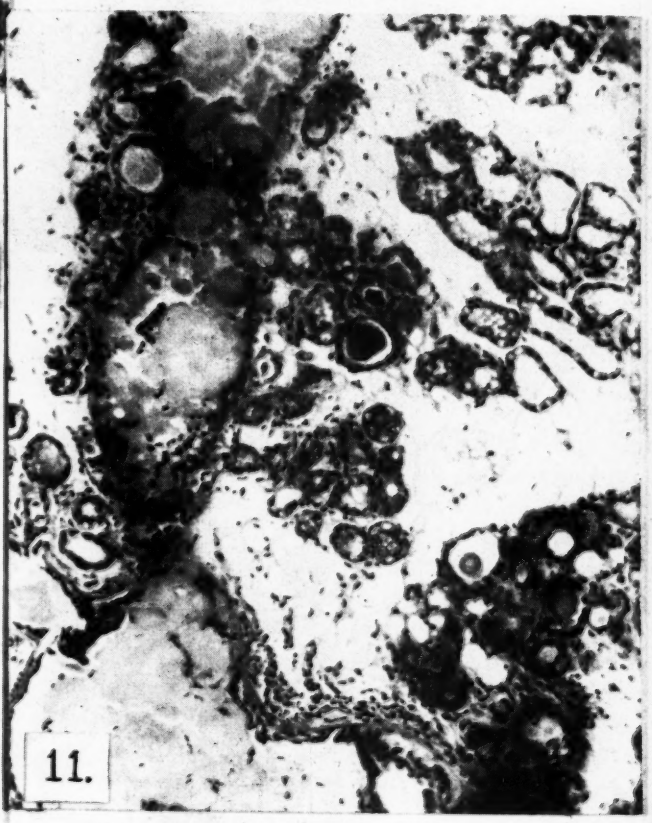
FIG. 5.—Group of acidophiles, chromophobes and a basophile from a normal section of an abnormal gland. From hypophysis of a 17 months old gonadectomized male (dba♀ × ce♂) F_1 hybrid WK 431. Mag. ×439.

FIG. 6.—Hemorrhagic cysts lined by abnormal basophiles in the pituitary of a 23 months old gonadectomized male (ce♀ × dba♂) F_1 hybrid WK 615. (Photomicrograph made from lantern slide, original negative lost, magnification unknown since laboratory fire.)

FIG. 7.—Hypertrophied basophiles; note enlarged negative image of the Golgi apparatus and large nucleoli. No other cell types present in this section. From 17 months old gonadectomized male (dba♀ × ce♂) F_1 hybrid WK 431. Mag. ×439.







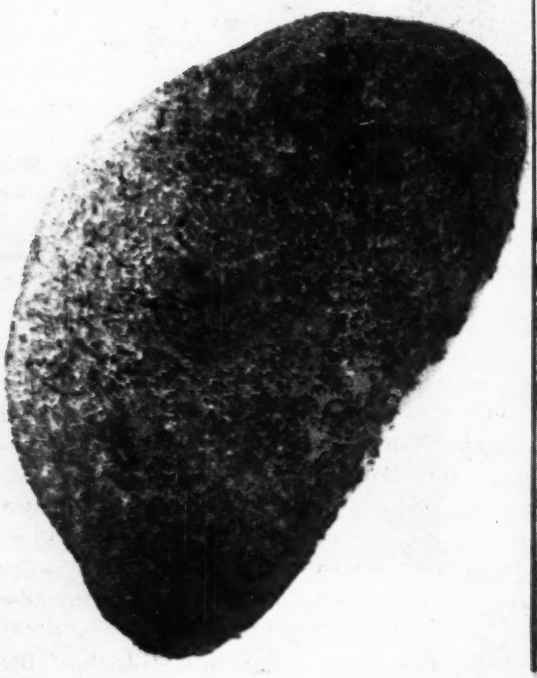


FIG. 8.—Region of abnormal basophiles showing loss of cohesion of these cells. From WK 431. Mag. ×439.

FIG. 9.—Cyst lined by ciliated epithelium at the periphery of the anterior lobe of the pituitary of an 18 months old gonadectomized female (ce♀ × dba♂) F_1 hybrid WK 448. Mag. ×132.

FIG. 10.—Section of abnormal pituitary showing enlarged sinuses. Section is composed of transitional basophiles and hypertrophied basophiles. From WK 448. Mag. ×93.

FIG. 11.—Mammary gland of a 20 months old gonadectomized male (dba♀ × ce♂) F_1 hybrid WK 516. Note secretion globules in the alveolar cells and ducts distended with secretion. Mag. ×132.

months old control female F_1 (C57 Black ♀ × dba ♂).[1]

MICROSCOPIC OBSERVATION OF THE MAMMARY GLANDS

The mammary glands of most of the experimental animals with hypophyseal tumors were examined histologically and showed alveolar cells and alveoli filled with secretion and the ducts distended with secretion (Figs. 11 and 12). In some of the glands there were areas of hyperplastic nodules (so-called precancerous lesions) and in four castrate males and a few gonadectomized females, mammary gland carcinomas were found. The mothers of these F_1 animals were dba strain mice. These mice had the milk factor from strain dba, and therefore with proper endocrine stimulation the mammary tumors might be expected, at least in intact animals. In other experimental animals where the milk factor was absent, the glands were well developed, but hyperplastic areas (so-called precancerous lesions) and mammary tumors never occurred.

In studying the mammary glands of castrate animals where no pituitary abnormalities occurred, there was no alveolar development observed. The amount of growth up to the stage of alveolar formation of these glands depended on the extent of abnormality occurring in the adrenals.

DISCUSSION

The results show that these abnormal pituitaries present several significant facts. The first and perhaps most important, is that in all cases the adrenal cortical tumors preceded the appearance of the gross hypophyseal tumors. Second, two types of nodules or tumors were present and both types were composed of basophiles, and third, the pituitary alterations were correlated with physiological changes in the animals that were manifested particularly in the mammary glands.

Very few spontaneous hypophyseal abnormalities have been reported previously in mice. Most of the abnormalities that have been reported were found to be disturbances of chromophobe cells and were observed following treatment with estrogens (5, 6, 16). Many spontaneous chromophobe cell adenomas have been found and studied in rats of advanced age (37, 40), and following treatment with various hormones (36, 38, 39).

For purposes of differentiating the present series of tumors of the anterior pituitary from chromophobe adenomas that have been reported, it might be said first of all that we are dealing with a different endocrine background than that used in the study of chromophobe adenomas. Here the hormonal stimulation arises from adrenal cortical hormones in castrate animals. Chromophobe adenomas have been observed both in intact old mice and rats and following estrogenic treatment in both species. Chromophobe adenomas produce a condition stimulating hypopituitarism when they occur following estrogen treatment, while in this series, from the physiological condition of the animal as expressed in the accessory reproductive organs, a state of hyperfunction exists. Part of the hormonal stimulation is directly attributable to the hormones secreted by the adrenal tumors but the extreme alveolar development of the mammary glands seems to depend upon the presence of the abnormal basophilic cells in the pituitary.

Reasons for believing that these various types of cells found in the abnormal areas of the pituitaries were basophiles are manifold. Their staining reaction with aniline blue showed that they had a great affinity for that dye. The negative image of the Golgi apparatus, which is quite different in normal acidophiles and basophiles, was always like that found in mature basophiles even though at times it was greatly hypertrophied. This hypertrophy indicates a heightened activity of the cell (15). There was obviously great physiological activity from the intense alveolar development of the mammary glands. Severinghaus states that with chromophobe adenomata there is no physiological alteration in the animal except that caused by de-

TABLE 2

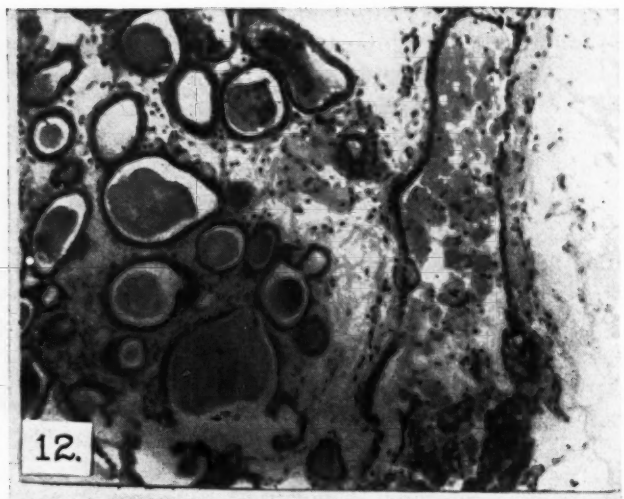
OCCURRENCE OF TWO TYPES OF CYSTS IN THE HYPOPHYSES OF GONADECTOMIZED AND CONTROL INTACT F_1 RECIPROCAL HYBRID MICE*

Sex	Number of animals	Ciliated cysts No.	Ciliated cysts %	Non-ciliated cysts No.	Non-ciliated cysts %	Total of all cysts No.	Total of all cysts %
⚲	186	14	7.52	23	12.36	37	19.88
⚲	178	7	3.94	19	10.67	26	14.61
Total ⚲ & ⚲	364	21	5.76	42	11.54	63	17.30
♀	213	7	3.28	14	6.57	21	9.85
♂	211	14	6.64	43	20.38	57	27.02
Total ♀ & ♂	424	21	4.95	57	13.44	78	18.39

* There is little difference in the percentage occurrence between the gonadectomized and control groups, but there is a statistically significant sex difference in the occurrence of non-ciliated cysts in the control F_1 hybrid mice.

struction of the anterior lobe and invasion of adjacent tissues. Furthermore, basophile cell disturbances are almost invariably associated with changes in the adrenal cortex (30).

Another factor in favor of classifying the present series of pituitary abnormalities as basophile



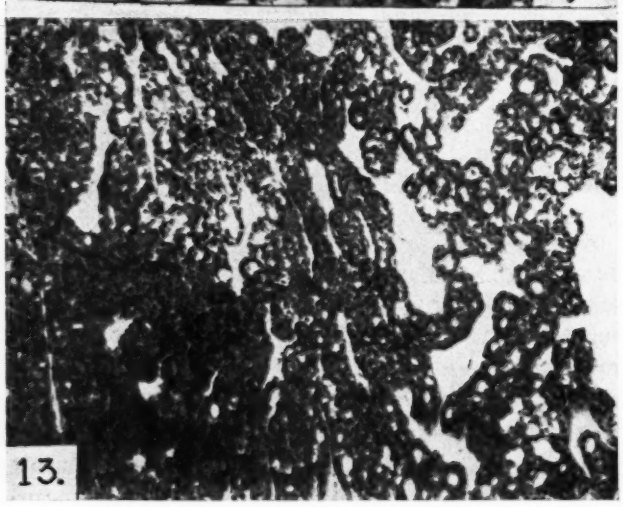


FIG. 12.—Mammary gland of 18 months old gonadectomized female (dba ♀ × ce♂) F_1 hybrid WK 461. The alveolar spaces and the ducts are distended with secretion. Mag. ×132.

FIG. 13.—Mammary gland adenoma (at right) and carcinoma (at left) from a 16 1/2 months old gonadectomized male (dba ♀ × ce♂) F_1 hybrid WK 425. In the adenomatous area there is some secretion in the alveoli. Mag. ×93.

disturbances is the size of the tumors. Where chromophobe adenomas occur they are described as large, filling the entire base of the skull and exerting pressure on the brain (16, 40, *et al.*). However, the tumors described here and those basophile adenomas reported elsewhere (17) have never attained this great size. They are discrete nodules not of sufficient volume to exert great stress on surrounding tissues. They have been observed to interrupt the normal contour of the intermedia and very occasionally of the posterior lobe.

The nodules found in the pituitaries of these mice were non-capsulated, but no invasion of other lobes or surrounding tissues was observed. Mitoses were rare, but evidences of amitotic division were prominent, a condition thought to be nonexistent in a normal hypophysis. By calling this condition amitotic division the authors are merely calling attention to the fact that the multinucleate cells were dividing in this manner or mitoses were incomplete and the cytoplasm did not divide. Another supposition is possible, and that is that mitosis was occurring too rapidly to permit the cytoplasm to divide. Selye has formed this opinion from studying the pituitaries of rats after hormone treatment (28).

The large transitional basophiles were in orderly acinar groups, while the small transitional cells and the abnormal basophiles were in dense clusters. These small cells may have, as Severinghaus states, passed a phase of hypersecretory activity and have become exhausted. The nodules formed by large transitional basophiles and small transitional cells follow closely Ewing's description of diffuse hyperplasia in the pituitary in man.

"On sections the growth may be diffuse . . . or focal adenomas may appear as opaque spots. . . ." "The arrangement of cells is orderly and mitoses are rare. While the gland usually remains solid, small cysts filled with colloid may develop and hemorrhages may occur" (12).

The relations of cell types and various hormones have been established. Acidophiles are associated with growth-promoting factors; these cells are said to be absent in dwarf mice (33) and there is an overabundance of them in acromegalics, in man (17). Pregnancy affects both acidophiles and basophiles (30). Basophiles are modified by thyroidectomy (31), states of thyroxine deficiency (18), and thyroid hyperplasia and adenomata (19). Also, evidence has been secured that basophiles secrete the adreno-corticotropic hormone (25). Nowhere as yet in the present series have been found morphological differences correlated with the presence of these pituitary tumors other than the unusual development of the mammary glands. From the literature very little reference was found, however, to mammary gland growth allied with pituitary disfunction. It has usually been modified by steroid hormone injection. It is known that mammary development follows treatment with some steroid hormone preparations, and in certain strains chromophobe adenomas occur. It is reasonable to assume that the development of the mammary glands is due to the interaction of the hor-

mone with various pituitary factors, or the chromophobe adenomas are transitional basophile types and hence would secrete the factors necessary for the mammary gland growth or at least part of its growth. Evidence against the latter is the difference in tumor size, and in spontaneous chromophobe adenomas no physiological action on the accessory reproductive system has been recorded. That this is true is shown by our observations that with the presence of the hypophyseal nodules, the excessive mammary gland development was the constant feature. The mammary gland development depends in part on the release of gonadotropic factors (34), a reason for believing that the substances associated with the abnormal basophiles are gonadotropin-like.

In parabiotic experiments, where a castrated rat was joined to an intact rat, it has been observed that there is an increase in the number of basophiles in the pituitaries of both animals, interpreted as storage of gonadotropic hormones (27, 35).

Hypertrophy of the Golgi bodies, and degranulation occur; these latter indicate an increase in secretion and its release (35). Thus it might be assumed with pituitary basophilism, that: (1) the storage and release of gonadotropins is greatly increased. A greater production of gonadotropins might affect the mammary glands in these experimental mice particularly since the gonads are not present and the hormones from the adrenals may either not regulate the gonadotropic action of the pituitary as the gonads do, or (2) the adrenal cortical secretions may be slightly different from those of a normal gonad, or (3) the pituitary secretions may act directly.

It is not the purpose of the authors to discuss the hormonal aspects involved in the present series at this time, since the substances elicited from the adrenal cortical tumors have not yet been identified, but to say briefly that the substance or substances elicited from the pituitary and adrenal glands seem to be of a gonadotropic and gonadal nature respectively. The effects produced on the mammary glands and accessory reproductive organs are similar to the effects produced by the hormones from the ovaries or combinations of ovaries and testes when they are present. It is well known that there are close relationships between the hypophysis and gonads and the hypophysis and adrenals. Recently much evidence has been reported on the interrelation of the pituitary and the mammary glands through the action of mammogenic hormones that affect the duct extension and lobule alveolar growth (23, 26). Gardner has stated that the hypophysis and the mammary glands are so closely related that the mammary gland development cannot be discussed unless the role of the pituitary is taken into consideration (16).

The presence of mammary tumors in certain of these animals with anterior pituitary tumors and also in the parental strain dba would seem to establish for gonadectomized animals the factors necessary for mammary tumor production. It is known that in intact animals the proper genetic constitution (such as in strains dba or C3H), hormonal influences and Mammary Tumor Inciter (M.T.I.) are necessities for spontaneous mammary tumor appearance generation after generation (3). Now in these animals under discussion, it is possible to postulate that the genetic constitution and

TABLE 3

HYPOTHESIS TO SHOW THAT MAMMARY TUMOR PRODUCTION IN GONADECTOMIZED MALE AND FEMALE MICE IS DEPENDENT UPON FOUR* FACTORS

Strain	Genetic constitution	Adrenal tumors (Hormonal influence)	M.T.I.†	Pituitary tumors	Mammary tumors
dba	+	+	+	+	+
ce	−	+	−	+	−
C57 Black	−	−	−	−	−
dba ♀ × C57Black ♂	+	+	+	±	−
C57Black ♀ × dba ♂	+	+	−	±	−
dba ♀ × ce ♂	+	+	+	+	+
ce ♀ × dba ♂	+	+	−	+	−
ce ♀ × C57Black ♂	−	+	−	+	−
C57Black ♀ × ce ♂	−	+	−	+	−

* The genetic constitution of the animal, the hormonal influences which are supplied by the adrenal tumors, the presence of mammary tumor inciter, and the presence of basophilic pituitary tumors.

† Mammary tumor inciter.

M.T.I. are still necessary and the hormonal influences that were supplied by the gonads are now supplied by the abnormal hypophyses and adrenals, to prepare the mammary glands for tumor development (Table 3). Further, that where the pituitary tumors are present the mammary glands are better prepared than with adrenal-cortical tumors alone.

In the F_1 reciprocal hybrid mice where the pituitary abnormalities were very infrequent, it is evident that the only change other than the pituitary tumor is the excessive mammary gland development. Even though other animals in these hybrid groups had adrenal tumors, the extensive mammary development appeared only when the pituitary was abnormal.

The pituitary cell changes in some respects are similar to those described by Crooke (7), Severinghaus (30), and Mellgren (25), in the pituitaries of patients with Cushing's syndrome. Basophilia has

been known to occur as a part of this and other syndromes. McLetchie (24) has stated that the castrate condition and Cushing's syndrome have much in common as in the latter there is frequently gonadal atrophy. Selye reports that it is very probable that in these cases tumorous hypophyses are capable of producing an excess of such pituitary hormones as adrenotropic, mammotropic, lactogenic and perhaps other factors (29). McLetchie declares further that:

". . . it is the rule rather than an exception to find adreno-cortical hyperplasia associated with basophil adenoma and again in some cases of adreno-cortical carcinoma, basophilia, that is the relative increase in basophil cells, is present . . . hypersecretory processes of the basophile cells and the adrenal cortex are complimentary, the one producing the other" (24).

In the adreno-genital syndrome, and in intersexuality, it has been thought that the pituitary becomes abnormal first and then the adrenal changes follow (4). In Addison's disease however, the adrenals become abnormal and the hypophyseal changes follow (8). In this experimental series the gross adrenal changes are primary. In some crosses adrenal cortical tumors occur as early as 8 months of age, while the hypophyseal changes are not grossly observed before 14 to 18 months in the dba × ce crosses and later in other crosses. It is possible to postulate that the pituitary may be thrown into unbalance primarily by castration, exert its effect on the adrenals in the absence of the gonads, and the adrenals in turn then become abnormal. The abnormal adrenals then secrete excess or unusual hormones which react on the pituitaries and they, then, become abnormal.

It seems pertinent to further compare the syndrome in mice with Cushing's syndrome in man.[4] This may well be an animal counterpart of Cushing's syndrome. The latter syndrome is a baffling problem both as to cause and therapy (9). Kepler (21) in an analysis of the present status of the problem has made several assumptions that might be applied to the mice. He states that the pituitary may become overactive primarily and cause the development of adrenal hyperplasia or neoplasia and then the abnormal adrenal cortices function excessively. If the abnormal basophilic cells are the primary cause of the adrenal disturbance, unless they produced only the adrenotropic agent and no other tropins, some manifestations should remain following removal of the adrenal tumors. Now, rather than suppose that the basophiles are secreting only one hormone, let us assume they are responsible for, and producing, at least one other tropin, one that affects mammary development since this seems to be the case in these animals. Woolley has transplanted an adrenal tumor from a ce mouse with a basophilic pituitary tumor and hyperplastic mammary glands. The transplanted adrenal tumor did not cause overdevelopment of the mammary glands in the new hosts, ce gonadectomized mice (44). It may be assumed that the pituitary tumor either directly or indirectly, rather than the adrenal tumor itself was responsible for the mammary gland growth. Here then, in mice at least is a factor that seems to be missing or that has taken another form of expression in man. The present situation then would be analogous to eosinophilic tumors in man (22) where there are other factors influenced by the anterior lobe cells. This would further advance the possibility that the pituitary is the primary site of the disturbances rather than the adrenals.

Castration in rats and mice causes increased gonadotropic potency. One might assume that the gonadotropins are transformed into a substance either identical with adrenocorticotrophic hormone (ACTH) or similar to ACTH that can act on the adrenals. Other workers have given serious consideration to the idea that since one cell type seems to produce so many hormones, the cell types may in effect secrete one or two basic precursors (17) that act in various ways according to the demands made upon it by other glands.

It is felt however, that the investigation of these tumors is by no means complete. Since there is a gross macroscopic change and histological change, perhaps there are identifiable biochemical changes also. Too, the developmental stages need to be studied more completely.

No cell types have been identified as exact counterparts of Crooke's change, the hyalinization of the basophile cells. It might be speculated that the large and often multinucleate abnormal cells in these mice are similar to the hyaline basophiles or Crooke's cells, but the hyalinization in the basophiles of the pituitaries of the mice is less acute, if present at all. These abnormal basophile cells in the mice compare more closely with the hypertrophic amphophiles described by Mellgren and others (25). Mellgren believes that these cells, the amphophiles, are later stages in the degranulation and hyalinization of the basophile cells. This might be the case in the mouse pituitaries since these anterior pituitary abnormalities seem to be in well-advanced stages of alteration at the time of autopsy. Collection of the early stages was not possible in this series because the experiments were well advanced at the time the pituitary abnormali-

[4] Cushing's syndrome indicates only those cases in which an adrenal tumor is present.

ties were first noted macroscopically. It is hoped that more information on analogies and the developmental processes will be learned by a study of pituitaries of younger gonadectomized animals.

From the preceding discussion, it is very evident that while the abnormalities of the pituitaries are a fact, their function, *i.e.* the function of the cells composing the abnormalities, with respect to the secretions they may produce is very speculative. In classifying the abnormalities as basophile disturbances, it is realized that this designation may be controversial. However, on the basis of effects on the mammary glands of the animals and the discussion of pituitary adrenal relationships, this classification is, at present, justified.

SUMMARY

Mice of the F_1 generation reciprocal crosses of JAX strains C57 Black, dba, ce, A and C3H were gonadectomized at 1 to 3 days of age. Virgin females and unmated males were used as control animals. At autopsy, from 14 to 26 months in the experimental groups, pituitary abnormalities occurred. Adrenal cortical carcinomata were also present. The highest incidence of hypophyseal abnormalities occurred in reciprocal hybrids of strains dba and ce. Histological analysis of the hypophyses showed that they were hyperplastic and some were adenomatous. These changes were considered to involve the basophile chiefly and it was decided that the abnormalities were basophilia (possibly adenomas) and focal adenomas. The most striking change, concurrent with the hypophyseal tumors, was the extensive differentiation and over-development of the mammary glands. This change became a criterion for predicting the presence or absence of the hypophyseal changes.

The possibilities as to hormonal activity of the hyperplastic and adenomatous areas of the pituitary were discussed and it was thought that in the combination of pituitary-adrenal disfunction, substances were secreted similar to the gonadotropic and gonadal hormones that reacted most differentially on the mammary glands. The pituitary tumors were compared to Cushing's syndrome in man. The pituitary response, *i.e.* the appearance of the tumors was, to some extent, a strain-limited factor, as is the pituitary response to hormonal injection.

It was postulated that although the gross pituitary changes were secondary to the changes observed in the adrenal, the fundamental change might have occurred first in the pituitary, reacted on the adrenals, and secretions emanating from the adrenals further reacted on the hypophysis so that gross changes occurred eventually after the adrenals were tumorous.

REFERENCES

1. ADDISON, W. H. F. The Cell Changes in the Hypophysis of the Albino Rat after Castration. J. Comp. Neurol., **28**: 441–463, 1917.
2. BIGGART, J. H. The Hypophysis of the Human Castrate. Bull. Johns Hopkins Hosp., **54**:157, 1934.
3. BITTNER, J. J. Possible Relationship of the Estrogenic Hormones, Genetic Susceptibility, and Milk Influence in the Production of Mammary Cancer in Mice. Cancer Research, **2**:710–721, 1942.
4. BROSTER, L. R., ALLEN, C., VINES, H. W. C., PATTERSON, J., GREENWOOD, A. W., MARRIAN, G. F., and BUTLER, G. C. The Adrenal Cortex and Intersexuality. Chapman and Hall Ltd., 1938.
5. BURROWS, H. Pituitary Hyperplasia in a Male Mouse after the Administration of Oestrin. Am. J. Cancer, **28**: 741–745, 1936.
6. CRAMER, W., and HORNING, E. S. Experimental Production by Oestrin of Pituitary Tumors with Hypopituitarism and of Mammary Cancer. Lancet, **230**:247–249, 1936.
7. CROOKE, A. C. A Change in the Basophil Cells of the Pituitary Gland Common to Conditions which Exhibit the Syndrome Attributed to Basophil Adenoma. J. Path. and Bact., **41**: 339–349, 1935.
8. CROOKE, A. C., and RUSSELL, D. S. The Pituitary Gland in Addison's Disease. J. Path. and Bact., **40**:255–283, 1935.
9. CUSHING, H. The Basophil Adenomas of the Pituitary Body and their Clinical Manifestations. Bull. Johns Hopkins Hosp., **50**:137, 1932.
10. DELAFIELD and PRUDDEN. Textbook of Pathology (revised by F. C. Wood), p. 1178, 13th ed. New York: Wm. Wood & Co., 1925.
11. DICKIE, M. M. A New Differential Stain for the Mouse Pituitary. Science, **100**:297–298, 1944.
12. EWING, J. Tumors of the Hypophysis. Neoplastic Diseases, pp. 978–1001. Philadelphia and London: W. B. Saunders Co., 1928.
13. FEKETE, E. Chapter III—Histology, Biology of the Laboratory Mouse, G. D. Snell, Ed. Philadelphia: The Blakiston Company, 1941.
14. FEKETE, E., WOOLLEY, G. W., and LITTLE, C. C. Histological Changes following Ovariectomy in Mice. I. dba High Tumor Strain. J. Exper. Med., **74**:1–8, 1941.
15. FOSTER, C. L. The Effect of Implanting Tablets of Synthetic Oestrogens on the Histology and Cytology of the Anterior Pituitary of Immature Rats. J. Endocrinology, **3**:79–86, 1942.
16. GARDNER, W. U., and STRONG, L. C. Strain-limited Development of Tumors of the Pituitary Gland in Mice Receiving Estrogens. Yale J. Biol. and Med., **12**:543–548, 1940.
17. GORMAN, W. J. The Endocrine Effects of Pituitary Tumors. Endocrinology of Neoplastic Diseases—A Symposium. G. H. Twombly and G. T. Pack, Eds. pp. 63–67. Oxford University Press, 1947.
18. GRIESBACH, W. E., and PURVES, H. D. The Significance of the Basophile Changes in the Pituitary Accompanying Various Forms of Thyroxine Deficiency. Brit. J. Exper. Path., **26**:13–17, 1945.
19. GRIESBACH, W. E., KENNEDY, T. H., and PURVES, H. D. Studies on Experimental Goitre. VI. Thyroid Adenomata in Rats on Brassica Seed Diet. Brit. J. Exper. Path., **26**:18–24, 1945.
20. GUYER, M. F., and CLAUS, P. E. Vacuolation of the An-

terior Pituitary Gland following Castration, Implantation of Cancer Tissue and Thyroidectomy. Anat. Rec., **67**:145–156, and Suppl. No. 2, 1937.

21. Kepler, E. J., Sprague, R. G., Clagett, O. T., Power, M. H., Mason, H. L., and Rogers, H. M. Adrenal Cortical Tumor Associated with Cushing's Syndrome. Report of a Case with Metabolic Studies and Remarks on Pathogenesis of Cushing's Syndrome. J. Clin. Endocrinol., **8**:499–531, 1948.
22. Kepler, E. J. The Relationship of "Crooke's Changes" in the Basophilic Cells of the Anterior Pituitary Body to Cushing's Syndrome (Pituitary Basophilism). J. Clin. Endocrinol., **5**:70–75, 1945.
23. Lewis, A. A., and Turner, C. W. Mammogenic Hormones of the Anterior Pituitary. I. Duct Growth Factor. Res. Bull., 310, U. of Missouri Coll. Agric. Exper. Sta., 1939.
24. McLetchie, N. G. B. The Pituitary Basophilism Syndrome of Harvey Cushing. J. Endocrinol., **3**:332–339, 1944.
25. Mellgren, J. The Anterior Pituitary in Hyperfunction of the Adrenal Cortex. Acta path. microbiol. Scandinav., Suppl. LX, 1944.
26. Mixner, J. P., Lewis, A. A., and Turner, C. W. Evidence for the Presence of a Second Mammogenic (Lobule-Alveolar) Factor in the Anterior Pituitary. Endocrinology, **27**:888–892, 1940.
27. Pighini, G., and Paoli, M. The Endocrine Glands of Rats in Parabiosis. Med. Jour. Rec., **124**:353–358, 7399–7404, 8474–8475, 1926.
28. Selye, H. Atypical Cell Proliferation in the Anterior Lobe Adenomas of Estradiol Treated Rats. Cancer Research, **4**:349–351, 1944.
29. Selye, H. Experimental Investigations Concerning the Role of the Pituitary in Tumorigenesis. Surgery, **16**:33–46, 1944.
30. Severinghaus, A. E. Anterior Hypophyseal Cytology in Relation to the Reproductive Hormones. Sex and Internal Secretions. pp. 1045–1087. 2d ed. Allen, Danforth and Doisey, Eds. Baltimore: Williams & Wilkins Co., 1939.
31. Severinghaus, A. E., Smelser, G. K., and Clark, H. M. Anterior Pituitary Changes in the Adult Male Rat following Thyroidectomy. Proc. Soc. Exper. Biol. and Med., **31**:1127–1129, 1934.
32. Slye, M., Holmes, H. F., and Wells, H. G. Intracranial Neoplasms in Lower Animals. Am. J. Cancer, **15**:1387–1400, 1921.
33. Smith, P. E., and MacDowell, E. C. An Hereditary Anterior-Pituitary Deficiency in the Mouse. Anat. Rec., **46**:249–257, 1930.
34. Turner, C. W. Sex and Internal Secretions. The Mammary Glands. pp. 740–807. 2d ed. Allen, Danforth and Doisey, Eds. Baltimore: Williams & Wilkins Co., 1939.
35. Witschi, E., and Levine, W. T. Oestrus in Hypophysectomized Rats Parabiotically Connected with Castrates. Proc. Soc. Exper. Biol. and Med., **32**:101–107, 1934.
36. Wolfe, J. M., and Brown, A. D. Action of Diethylstilbesterol on Cytological Characteristics of Anterior Pituitaries of Female Rats, Together with Certain Observations on the Effect of Castration. Endocrinology, **31**:467–478, 1942.
37. Wolfe, J. M. The Effects of Advancing Age on the Anterior Hypophysis and Ovaries of Female Rats. Am. J. Anat., **72**:361–383, 1943.
38. Wolfe, J. M., Wilson, J. G., and Hamilton, J. B. The Effect of Early Postnatal Injections of Testosterone Propionate on the Structure of the Anterior Hypophyses of Male and Female Rats. Yale J. Biol. and Med., **17**:341–349, 1944.
39. Wolfe, J. M., and Wright, A. W. Histologic Effects Induced in the Anterior Pituitary of the Rat by Prolonged Injection of Estrin with Particular Reference to the Production of Pituitary Adenomata. Endocrinology, **23**:200–210, 1938.
40. Wolfe, J. M., Bryan, W. R., and Wright, A. W. Histologic Observations on the Anterior Pituitaries of Old Rats with Particular Reference to the Spontaneous Appearance of Pituitary Adenomata. Am. J. Cancer, **34**:352–372, 1938.
41. Woolley, G. W., and Little, C. C. The Incidence of Adrenal Cortical Carcinoma in Gonadectomized Female Mice of the Extreme Dilution Strain. I. Observations on the Adrenal Cortex. Cancer Research, **5**:193–202, 1945.
42. Woolley, G. W., and Little, C. C. The Incidence of Adrenal Cortical Carcinoma in Gonadectomized Female Mice of the Extreme Dilution Strain. II. Observations on the Accessory Sex Organs. Cancer Research, **5**:203–210, 1945.
43. Woolley, G. W., and Little, C. C. The Incidence of Adrenal Cortical Carcinoma in Gonadectomized Male Mice of the Extreme Dilution Strain. Cancer Research, **5**:211–219, 1945.
44. Woolley, G. W., and Little, C. C. Transplantation of an Adrenal Cortical Carcinoma. Cancer Research, **6**:712–717, 1947.
45. Woolley, G. W., and Little, C. C. Prevention of Adrenal Cortical Carcinoma by Diethylstilbesterol. Proc. Nat. Acad. Sc., **32**:239–240, 1946.
46. Woolley, G. W., Unpublished Data.

CORRECTION

The title of the last column of Table I in the paper by Bass and Feigelson, Cancer Research, **8**: 507, October 1948, should read "Dry weight mean" instead of "Wet weight mean."